IN VITRO MODELS OF HUMAN LUNG EPITHELIAL CELL DIFFERENTIATION

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In Vitro Models of Human lung Cell Differentiation

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I hereby certify that this material, which I now submit for the assessment on the program of study leading to the award of Ph.D. is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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Date: 17/01/00

This thesis is dedicated to my father and
to the memory of my mother.

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ABBREVIATIONS

ATCC	American Tissue Culture Collection
BrdU	5-Bromo-2'-Deoxyuridine
CAM	Cell Adhesion Molecule
cDNA	complementary Deoxyribonucleic Acid
CEA	Carcinoembryonic antigen
CGR	Calcitonin Gene-Related Protein
CK	Cytokeratin
CRABP	Cytoplasmic Retinoic Acid-Binding Protein
CRH-KO	Corticotropin-Releasing Hormone-knock out
DMEM	Dulbecco's Minimum Essential Medium
DMSO	Dimethyl sulfoxide
DNase	Deoxyribonuclease
E-CAD	E-Cadherin
ECM	ExtraCellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGF-R	Epidermal Growth Factor Receptor
EGP	Epithelial Glycoprotein
ER	Endoplasmic Reticulum
ES cells	Embryonic Stem Cells
ESA	Epithelial Specific Antigen
FAK	Focal Adhesion Kinase
FCS	Fetal Calf Serum
FGF	Fibroblast Growth Factor
FGF	Fibroblast Growth Factor
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
HLA-DR	Human Leukocyte Antigen-DR
HSM	Hormone supplemented medium

IC_{50}	Inhibitory Concentration 50%
I-CAM	Intercellular Adhesion Molecule
IgE	immunoglobulin E
IGF-1	Insulin-like Growth Factor - 1
IGF-R	Insulin-like growth factor receptor
kDa	Kilodalton
MAb	Monoclonal Antibody
MAP-K	Microtubual associated protein
MEM	Minimum Essential Medium
min	Minute(s)
MMLV-RT	Moloney Murine Leukemia Virus-Reverse Transcriptase
mOsm/kg	Milli - Osmoles per Kilogram
mRNA	Messenger RNA
N-CAD	N-Cadherin
N-CAM	Neural Cell Adhesion Molecule
NCTCC	National Cell & Tissue Culture Centre
NEAA	Non-Essential Amino Acids
NGF	Nerve Growth Factor
NSCLC	Non-Small Cell Lung Carcinoma
P	Passage
PBS A	Phosphate Buffered Saline A
PDGF	Platelet Derived Growth Factor
PVP	Polyvinylpyrrolidone
RA	Retinoic Acid
RAR	Retinoic Acid Receptor
RARE	Retinoic Acid Responsive Element
rER	Rough Endoplasmic Reticulum
RNase	Ribonuclease
rpm	Revolution(s) Per Minute
RT	Room Temperature
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
RXR	Retinoic X Receptor
SCLC	Small Cell Lung Carcinoma
SDS	Sodium Doedecyl Sulphate

sec	Second(s)
SMGC	Small Mucous Granule Cell
Sp-A	Surfactant protein-A
SP-B	Surfactant protein-B
TBS	Tris Buffered Saline
TDS cells	Tissue Determined Stem Cells
TEMED	N, N, N', N'-Tetramethyl-Ethylenediamine
TGF-α	Transforming Growth Factor α
TGF-β	Transforming Growth Factor β
TNF-α	Tumour Necrosis Factor - α
Tris	Tris(hydroxymethyl)aminomethane
v/v	volume/volume
V-CAM	Vascular Cell Adhesion Molecule
w/v	weight per volume

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ABSTRACT

Previous studies in this laboratory have demonstrated that BrdU induces differentiation in the lung epithelial cell lines DLKP (derived from a poorly differentiated carcinoma of the lung) and A549 (derived from an adenocarcinoma of the lung). This differentiation involves the induction of epithelial specific proteins, e.g. cytokeratins-8 and –18, and epithelial related adhesion molecules e.g. $\alpha_2\beta_1$ integrin. This thesis investigated the effects BrdU has on the expression of Ep-CAM, a non-calcium dependent, homophilic cell-cell adhesion protein, in these cell lines. BrdU treatment induced Ep-CAM protein expression in DLKP after 7 days, with this induction reaching a plateau after 14 days of 10μM BrdU treatment. Similarly, in A549, Ep-CAM was induced following 7 days of BrdU treatment and the level of induction also plateaued after 14 days of exposure to 10μM BrdU. RT-PCR analysis revealed that the effect of BrdU on Ep-CAM expression appears to be at the post-transcriptional/translational level, with no increase in mRNA levels compared to significant increase in protein levels in both cell lines.

Ep-CAM is believed to interfere with the functioning of the Ca^{2+}-dependent cell-cell adhesion molecule E-cadherin, by causing alterations in focal adhesion proteins. Analysis of two these proteins, α-actinin and α-catenin, showed that BrdU down-regulated their expression following Ep-CAM induction.

RT-PCR analysis of the Ep-CAM homologue GA733-1 revealed that, following BrdU treatment, mRNA expression is induced in DLKP. However, no induction is observed in A549.

The ability of other halogenated thymidine analogues to induce differentiation was also investigated. Three halogenated thymidine analogues were selected, each possessing a different mechanism of biological activity, CdU (which incorporates into DNA), 5,5'-FdU (which inhibits DNA synthesis) and 5-BUr (which incorporates into RNA). The ability of CdU to induce the expression of cytokeratin-8, cytokeratin-18, cytokeratin-19, Ep-CAM and β1-integrin was demonstrated in both A549 and DLKP. RT-PCR analysis

of A549 revealed that although the protein expression was induced, the mRNA level remained unchanged indicating that CdU was altering expression at a post-transcriptional/translational level. A significant up-regulation of cytokeratin-8, cytokeratin-18, cytokeratin-19, Ep-CAM and β1 integrin also occurred in DLKP and A549 following 5,5'-FdU treatment. Analysis of mRNA levels following treatment with 5,5'-FdU indicated that expression was being altered at a post-transcriptional/translational level. Treatment of DLKP and A549 with 5-BUr did not produce any obvious alterations in protein expression or mRNA levels.

To develop models reflecting *in vivo* differentiation, DLKP and A549 were grown in a hormone supplemented medium (HSM) which contained a number of physiologically relevant factors e.g. oestrogen and insulin. Growth in this medium induced expression of cytokeratin-8, cytokeratin-18, cytokeratin-19, and Ep-CAM in DLKP and A549. Experiments to identify the importance of specific components in HSM revealed that the deletion of hydrocortisone, and cholera toxin from HSM cause an increase in induction of cytokeratin-19 and Ep-CAM. In contrast the removal of insulin from HSM, reduced the ability to induce expression of cytokeratin-19 and Ep-CAM in A549.

To further develop *in vitro* models reflecting *in vivo* differentiation, methods were established to generate primary cultures of lung tumour cells and normal lung epithelial cells. The assessment of a variety of methods for the isolation of lung carcinoma cells from lung tumour samples did not reveal any advantages between the methods. Preliminary studies on isolated normal rat type II pneumocytes revealed morphological and antigenic changes during *in vitro* cultivation. These changes were consistent with the terminal differentiation of type II pneumocytes into type I pneumocytes. During the isolation of lung tumour cells cultures of fibroblasts were often established and these expressed the unusual feature of cytokeratin protein expression, which is usually epithelial-specific.

1.0 INTRODUCTION.

1.1 CELL TYPES OF THE LUNG

The lungs are organised into the intrapulmonary airways (bronchi and bronchioles) which account for 6-10% of the lung volume, and the gas exchange area or parenchyma, which accounts for approximately 80-90% of the lung volume. The lung is encapsulated by the viseral pleura, a layer of connective tissue and mesothelial cells. The pleura, along with the nervous and vascular tissue associated with the lungs account for 9-10% of the lung volume. Thus, the lung is an extremely complex organ. This complexity means that the lung consists of over 40 different cell types, allowing the organ to function as the principle gas exchange in the body, with a surface area of $70m^2$. The lung also has a number of nonventilatory functions including humidification, thermal regulation, mucociliary clearance, anti-bacterial response, and elimination of volatile substances.

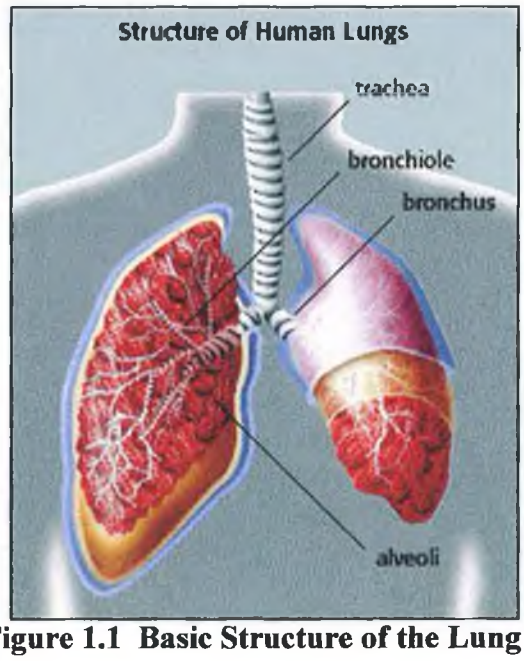


Figure 1.1 Basic Structure of the Lung

1.2 EPITHELIAL LUNG CELL TYPES

1.2.1 TRACHEOBRONCHIAL AIRWAYS.

At least seven epithelial cell types lining the tracheobronchial airways have been identified. These epithelial cells types are classified as follows: basal cells, ciliated cells, brush cells, goblet (or mucous) cells, serous cells, Clara cells, and neuroendocrine cells (the latter four function as secretory cells) (Crapo *et al.*, 1982).

Basal cells

Basal cells are oriented with long axes parallel to and in contact with the basal lamina. The cytoplasm, containing many filaments and few organelles, is usually small in relation to the nucleus. It is thought to be a progenitor cell for the other cell types (Plopper, 1996).

Ciliated cells

These are epithelial cells, which possess cilia of approximately 0.25μm in diameter. The function of these cilia is to sweep the mucous that lines the trachea to the oesophagus where it is swallowed and eliminated.

Goblet (or Mucous cells)

Goblet cells are columnar in shape but may become distended in shape due to their intracellular secretions. The cell cytoplasm is characterised by numerous secretory granules which contain high molecular weight mucous acidic glycoprotein and electron lucent granules.

Serous cells

Serous cells have basal, located nuclei and apical microvilli. They contain abundant rough endoplasmic reticulum (rER) and apical electron-dense sectretory granules. (Plopper, 1996)

Clara cells

Clara cells have characteristics of both sectretory cells and those capable of metabolising xenobiotic compounds. The secretory granules are discrete membrane bound electron dense structures that contain either neutral glycoprotein or low molecular weight protein such as cc10 (Singh and Katyal, 1992). They also produce surfactant proteins A and C. Clara cells have mitochondria scattered throughout their cytoplasm and a variable amount of a granualar ER and glycogen. The Clara cell is the predominant non-ciliated cell in all the generations of intra-pulmonary airways in laboratory animals (Plopper, 1996).

Neuroendocrine cells

Neuroendocrine cells are typically pyramid in shaped with their bases containing osmophilic granules, abundant ER, Golgi complex, ribosomes, and many filaments. These cells are most abundant in the early stages of life and are sometimes associated with nerve endings (Plopper, 1996).

Other epithelial cells

Other unidentifiable epithelial cells exist as a small population. They have none of the characteristics of the several cell types outlined previously and have a generalised cell structure (Plopper, 1996).

1.2.2 TRANSITION ZONE.

The transition zone, the area where small air passages of the tracheobronchial tree join the gas exchange area, is the focus of many lung disorders. Their histological appearance is similar to that of terminal bronchioles with the exception that the epithelium is interrupted by alveoli (Plopper, 1996).

1.2.3 PARENCHYMA.

Parenchyma (or respiratory area) consists of functional units called acini or terminal respiratory units. The acini are generally defined, as all the airspace's distal to one terminal bronchiole. This includes the respiratory bronchiole branching from it and all the associated alveolar ducts, alveolar sacs, alveoli, and the airspace's contained within these structures (Plopper, 1996). Two epithelial cell types line the interalveolar septa, type I pneumocytes and type II pneumocytes (Penny, 1988).

Type I Pneumocytes

Type I pneumocytes are squamous cells with a centrally placed nucleus and possess a large cytoplasmic volume (cellular surface area is 5000 to 7000μm^2). There are few organelles present in the cells ultrastructure e.g. mitochondria, only minimal amounts of rER are observed, and a moderate number of endocytoic vesicles are present. The type I pneumocyte is the cell through which the gas exchange process occurs. Type I pneumocytes cover approximately 96% of the alveolar surface yet only account for 45% of the cell mass in the alveoli (Plopper, 1996).

Type II Pneumocytes

Type II pneumocytes cover the remainder of the alveolar surface area (approximately 3%). Type II pneumocytes are cuboidal cells possessing microvilli, and contain many cellular organelles e.g. mitrochondria (accounting for 5-9% of cell volume), rER,

microvesicles, and Golgi complex. One of the main characteristic features of this cell type is the presence of many osmophilic, laminated vesicles called lamellar bodies. These vesicles are storage bodies for surfactant, a heterogeneous mixture of lipid, carbohydrate and protein that forms a highly surface active complex, of which type II pneumocytes are the main producers (Plopper, 1996). The main role of surfactant is the reduction of the surface tension at the air-liquid interface hence stabilising the alveoli and preventing lung collapse (Hollingsworth and Gilfillan, 1984). Other ancillary roles of the surfactant system include host defence, acting as an opsonisation agent for the ingestion of bacteria by macrophages (Guzman *et al.*, 1994), and the prevention of oedema in the alveoli spaces (Chevalier and Collet, 1972). The type II pneumocyte is also thought to act as a progenitor cell for both type I and type II pneumocytes (Sing and Katyal, 1992; Adamson and Bowden, 1975). Various studies both *in vivo* and *in vitro* have shown that type II pneumocytes must undergo a number of antigenic and morphological changes to adopt a type I cell phenotype (Paine *et al.*, 1995).

1.3 LUNG DEVELOPMENT.

The development of the lung requires cell proliferation, branching morphogenesis, alveolar saccule formation and cell differentiation. These processes require well co-ordinated events, which are achieved by epithelial-mesenchymal interactions, activation and repression of transcriptional factors, cytokine signalling, cell cycle control and extracellular matrix expression and signalling (see section 1.5).

The lung originates as a ventral appendage of the endodermal epithelium lining the floor of the primitive embryonic pharynx. It then divides laterally into two buds and begins dichotomous branching into the surrounding splanchnic mesenchyme. This repetitive epithelial branching process, termed branching morphogenesis, is characteristic of lung formation and continues throughout gestation.

Lung development can be divided into four chronological stages:

I) ***The pseudoglandular stage*** during which the bronchial and respiratory tree develops and an undifferentiated primordial system forms. It is during this stage that the most significant growth and branching of the primitive lung epithelium takes place. This determines the pattern of the lung system. The epithelial cells at this stage have a columnar morphology and are undifferentiated.

II) ***The canalicular stage*** during which the respiratory bronchioles emerge, and this is accompanied by the development of terminal sacs and vascularisation. Towards the end of this stage, the undifferentiated cuboidal cells begin to develop inclusion bodies, characteristic of type II pneumocytes and Clara cells.

III) ***The terminal sac*** stage during which the number of terminal sacs and vascularisation increases. During this stage differentiation of type II and type I pneumocytes occurs.

IV) ***The alveolar stage*** during which terminal sacs develop and enlarge into mature alveolar ducts and alveoli.

1.4 STEM CELL THEORY IN LUNG.

Stem cells are characterised by an ability for self-maintenance and to vary this self-maintenance (i.e. to proliferate without maturation) and also to generate a large number of differentiated functional progeny following injury (Potten and Loffer, 1990). It should be noted that while a differentiation potential is a property of stem cells it should not be confused as an essential feature of 'stem-ness'.

Although stem cells in adult organs are pluripotent, the ultimate differentiated lineage descendants are not usually expressed beyond the relevant organ in which the stem cell occurs, i.e. these stem cells are tissue determined stem cells (TDS cells) and are thus considered separate from embryonic stem cells (ES cells) (Sell, 1994). Another essential difference between TDS cells and ES cells is their differentiation status, all stem cells with the exception of those present in the zygote (i.e. ES cells) are differentiated to a greater or lesser extent. The progeny of TDS cells may undergo further differentiation thereby giving rise to further differentiated descendants.

These TDS cells often divide and differentiate to give rise to transit cells, which are still capable of division. It is the division and further differentiation of these transit cells that is responsible for increasing end cell number (Lajtha, 1982; Potten and Loffler, 1990). Transit cells are more mature than stem cells, often showing intermediate properties.

An important feature of transit cells is that they are inexorably destined to move towards functional status. This can be demonstrated using the following model (figure 1.4.1). If one stem cell is mobilised into differentiation and the transit cells it derives undergoes 10 cycles of amplification then just over 1,000 cells are produced. If one assumes it takes 24 hours for all cell proliferation (stem cell and transit cell) then a small population of cells can maintain a high-end cell number. This is the situation which applies to bone marrow, skin and intestinal epithelium (Lajtha, 1982).

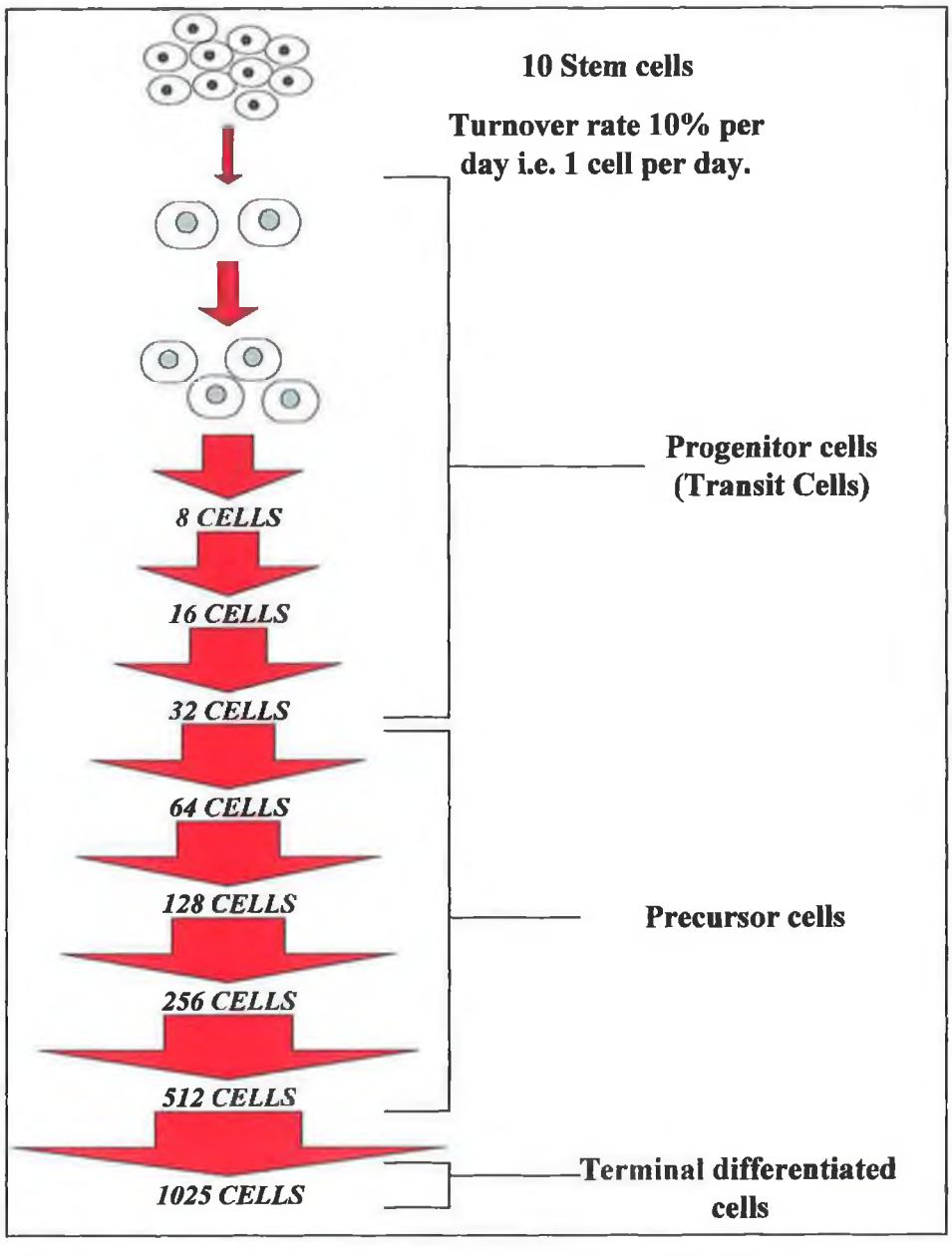


Figure 1.4.1 Demonstrating how a small population of TDS cells can maintain high cell populations in tissue.

TDS cells are believed to undergo a slow cell cycle in order to reduce the risk of errors during DNA replication. As TDS cells are present throughout the life of the organism, such error could become amplified in the organism (Lajtha, 1982). Indeed it is proposed that most tumours contain TDS cell populations (Khan *et al.*, 1991) and that the overlapping expression of differentiation markers (Gazdar *et al.*, 1988) within cancer cells is indicative of a stem cell origin for most lung epithelial tumours.

1.4.1 GENERAL MODELS OF DIFFERENTIATION IN STEM CELLS.

During the differentiation process of TDS cells, it is necessary that they maintain a constant cell number. One popular model for this is asymmetrical cell division. According to this model, when the stem cell divides one daughter cell remains a stem cell while the other becomes a transit cell and enters the differentiation process (Figure 1.4.2). This model suffers from a fundamental flaw in that it suggests that after receiving an appropriate stimulus it proceeds into a cell cycle that yields two different daughter cells (Lajtha, 1982).

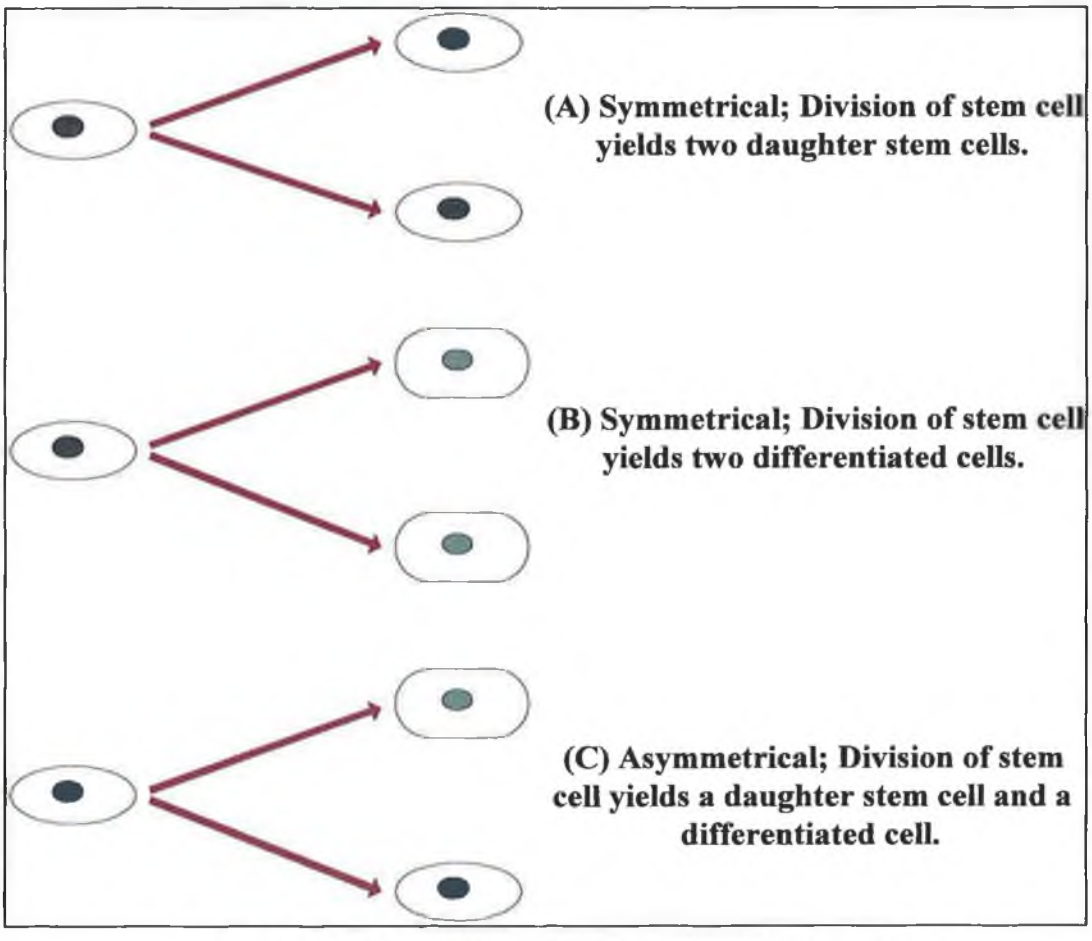


Figure 1.4.2 Models for stem cell fate following division.

Although proliferation and differentiation appear to be interlinked processes during stem cell maturation, they are quite separate events that occur concomitantly. This suggests the whole differentiation process may be understood in terms of a spiral model (Potten and Loffler, 1990).

Some TDS cells appear to be highly pluripotent giving rise to several different cell lineages, e.g. the haematopoietic system. Given this pluripotency, it can be envisaged that depending on the signal, a stem cell will adopt one direction of maturation over another.

According to a model proposed by Holtzer *et al.* (1975), TDS cells are only capable of binary decisions, e.g. stem cell A is capable of generating cells B or C, and cell B may then differentiate to cells D or E. As the transit cells pass through this hierarchy they go through cell division.

1.4.2 STEM CELL MODELS IN LUNG TISSUE.

The principle of stem cells and their *in vitro* cultivation and manipulation is now well established for a number of tissue types (see table 1.4.1).

Mammary gland	Rudland and Barraclough (1988)
Liver	Sell (1994)
Brian	Bartlett *et al.* (1995)
Hematopoietic tissue	Fraser *et al.* (1995)
Intestine	Hermiston and Gordon (1995)
Skin	Jones *et al.* (1995)

Table 1.4.1. *In vitro* cultivation of tissue stem cells.

The existence of a similar stem cell in the lung is strongly suspected, given the ability of the lung to regenerate when exposed to local damage by atmospheric components (e.g. smoke, carbon black particles) and by lipophilic chemicals absorbed (e.g. through the gut) into the blood stream. However, identification of such a stem cell is hampered by the complexity of the respiratory system and the variety of cell types present (Plopper and Hyde, 1992; Paine and Simon, 1996; Mariassy, 1992).

The most predominant hypothesis for stem cells *in vivo* in lung is that a different set of progenitor cells exist (including basal cells) each destined to give rise to a discrete differentiated cell type (Evans *et al.*, 1989; Jetten, 1991; Plopper *et al.*, 1992). In the case of type II cells, these cells proliferate and then differentiate into type I cells (Adamson and Bowden, 1979) and Clara cells can differentiate into ciliated cells (Jetten, 1991).

However, an alternative proposal is that there is a single pluripotent stem cell for generating ciliated cells, Clara cells and the other differentiated cell types (McDowell, 1987). This hypothesis suggests the existence of a monotypic stem cell, which gives

rise to a transit cell described as a small mucous granule cell (SMGC). This cell is defined as being of a secretory yet premature type containing a few small granules which are periodic acid schiff reaction positive, and also possess a well developed endoplasmic reticulum, prominent Golgi complex and occasional tonofilament bundles. It is believed to possess an electron microscopically pale cytoplasm with apex reaching the lumen, and appears to show no evidence of ciliary or mucous differentiation. This SMGC is believed to be able to arise through dedifferentiation of any differentiated secretory cell type. There is some empirical evidence for this hypothesis as Clara cells are found to be positive for surfactant protein-A (SP-A) and surfactant protein-B (SP-B) which were originally described as specific products of type II pneumocyte cells. This suggests that Clara cells and type II pneumocytes are derived from the same stem cell. Further tentative indications come from studies of fetal mouse lung where a definite population of progenitor cells co-express SP-A (type II cells), cc10 (Clara cells), and calcitonin gene-related protein (CGRP) (pulmonary neuroendocrine cells), each being a gene product used to indicate differentiated function in a different respiratory epithelial cell type (Wuenschell *et al.*, 1996).

In an attempt to reconcile these varying hypotheses, it is necessary to divide the respiratory system into two segments, the pseudostratified epithelium and the simple epithelium. In the proposed model, the same stem cell is likely to occur for both at the fetal stage and to remain in the epithelium through the whole peri- and post-natal stages of life (Figure 1.4.3). However, this stem cell at stage I does not appear to be involved in the steady state cell turnover but pursues its role to repopulate damaged, severely injured epithelia with various transit cells or predifferentiated secretory cell populations (stage II). These transit cells further differentiate into phenotypically functionally cell types (stage III). The majority of the differentiated cells present in stage III will differentiate irreversibly (stage IV) to their ultimate cell type. A small proportion of the differentiated cells remains at the stage III of the differentiation pathway. These cells at stage III are able to dedifferentiate into the presecretory or predifferentiated cells of stage II, thus participating in steady state turnover between stage II and stage III. In the fetal stage of differentiation, the process seems to proceed directly from stage I to stage III (Emura, 1997).

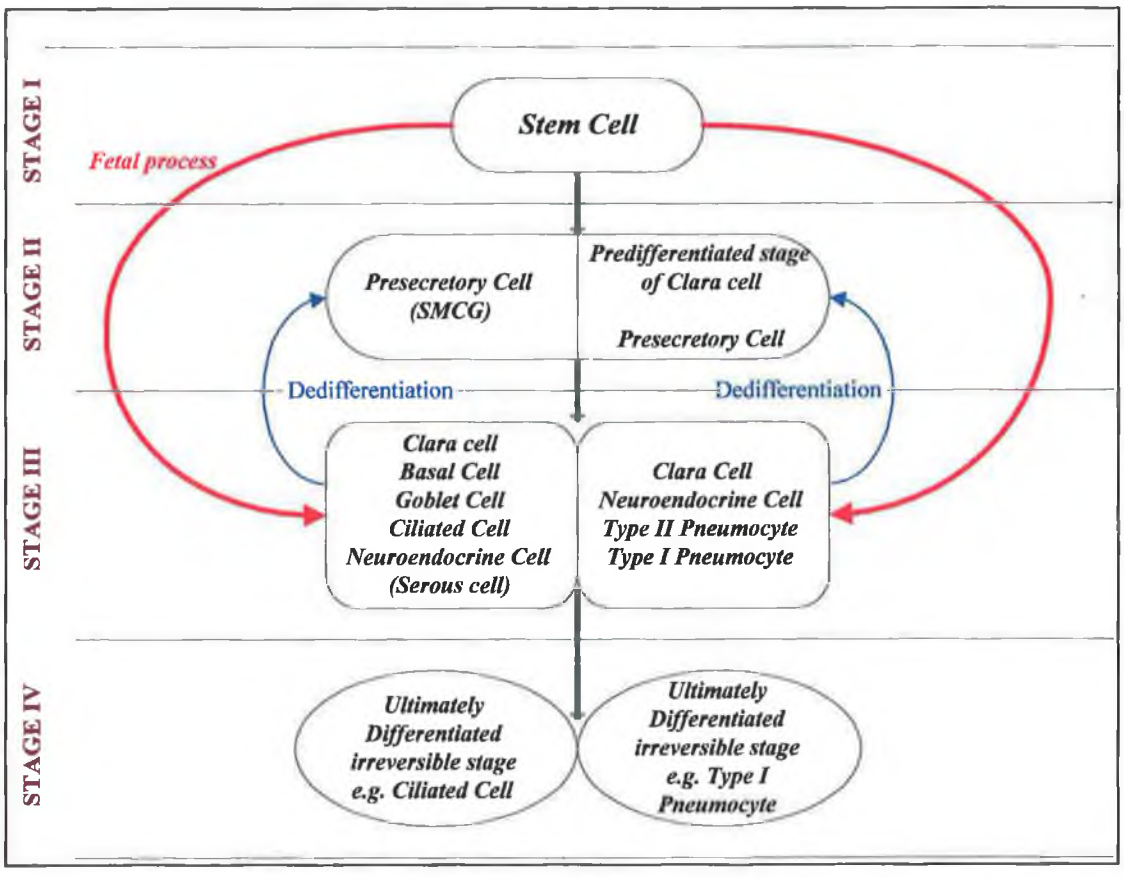


Figure 1.4.3 Proposed model for the generation and maintenance of epithelial cell lineages in lung.

The proliferation and differentiation of type II pneumocytes into type I pneumocytes is an established fact even if specific elements are still unknown. The existence of a population of stem cells for the whole lung is more controversial.

In summary, very little hard scientific data exists about stem cells in the lung, the pathways they follow, their distribution and mechanism of action. No markers yet exist for lung stem cells. The idea of dedifferentiation is in contrast to the stem cell models developed in skin, liver and intestine in which the stem cell pre-exist in the epithelium (Emura, 1997). The lung is susceptible to local damage from a number of sources. These include atmospheric components (e.g. ozone, silica, and carbon black particles); lipophilic chemicals absorbed (e.g. through the gut) into the blood stream, and viral and bacterial infection. Therefore, it must possess some form of mechanism to regenerate itself, even if limited. In attempting to identify if a cell is a stem cell, its native state is often altered during the investigation. This may result in loss of the

stem cell or only a limited spectrum of responses being observed from the cell. Thus, due to the variety of cell types present and by the complexity of the respiratory system, identification of a lung stem cell is a difficult task.

1.5 FACTORS CONTROLLING DIFFERENTIATION IN THE LUNG

1.5.1 PHYSIOLOGICAL AGENTS OF LUNG DIFFERENTIATION.

The physiological agents involved in the modulation of lung differentiation *in vivo* are largely unknown. A number of studies using isolated lung cells (both adult and fetal) and cell lines have implicated a number of candidate factors including glucocorticoid steroids, chemokines, cell-matrix and cell-cell interactions. The mechanisms and interaction of these molecules are at best poorly understood.

1.5.1.1 Glucocorticoid Steroids.

The glucocorticoid steroids regulate gene expression by binding a specific receptor in the cytoplasm. Upon binding, a hormone-receptor complex is formed with an increased affinity for DNA and migrates into the nucleus. After entering the nucleus, alterations in transcription of specific genes occur. The glucocorticoid steroids can also act in a post-translational level by increasing the number of ribosomes translating the messenger RNA (Palmiter, 1972).

The importance of the glucocorticoid steroids in lung development can be observed in Corticotropin-releasing hormone-deficient (CRH-KO) mice. A consequence of this mutation is that mice are glucocorticoid insufficient and exhibit neonatal lethality. Death is due to respiratory insufficiency as a result of abnormal pulmonary development, and in particular, impaired maturation of type II pneumocytes and Clara cells (Muglia *et al.*, 1999). *In vivo* studies have also implicated hydrocortisone in the development of the gas exchange area or the differentiation of type II alveolar cells (Kendall *et al.*, 1990).

Since glucocorticoids act in a systemic manner, often stimulating paracrine signals, it is therefore difficult to determine their exact role in a particular cell type. *In vitro* studies have helped to elucidate some of the mechanisms of action of glucocorticoid steroids in lung differentiation. Several studies have shown that the glucocorticoid steroids act on lung epithelial cells directly. For example, in studies using isolated type II pneumocytes and various cell line models such as A549, glucocorticoids induced functional differentiation as seen in the production of pulmonary surfactant, which they control at both transcriptional and post-transcriptional levels, and induction of alkaline phosphatase activity (a marker for type II pneumocytes) (Speirs *et al.*, 1991). However, the majority of glucocorticoid effects on lung epithelial cell differentiation (in both fetal and adult systems) appear to be through the mediation of soluble factors from glucocorticoid stimulated fibroblasts (Post *et al.*, 1984; Speirs *et al.*, 1991; McCormick *et al.*, 1995). In the case of oestrogen, for example, both *in vivo* and *in vitro* studies have found that it accelerates lung differentiation at the expense of lung growth (Khosla *et al.*, 1981; Adamson *et al.*, 1990). The action of oestrogen on lung epithelium is mediated in part, by fibroblasts binding oestrogen and subsequently transferring a maturation factor(s) to the fetal epithelium (Adamson *et al.*, 1990).

1.5.1.2 Chemokines.

A number of different soluble factors have been implicated in lung epithelial cell differentiation. Most of these are produced by lung fibroblast cells and by other lung cells and appear to act in an autocrine and paracrine manner.

The Fibroblast Growth Factor (FGF) family, which contains several different polypeptides, appears to have an important role in lung development, with fetal lung epithelium having been shown to be positive for FGF-Receptor (Han *et al.* 1992). *In vivo* and *in vitro* studies have implicated several members of the FGF family in lung growth and development in both fetal and adult tissue. These include acidic-FGF, basic-FGF, and KGF (FGF-7) (Lesur *et al.*, 1992; Leslie *et al.*, 1993; Ulich *et al.*, 1994).

Epidermal Growth Factor (EGF) has been implicated in general epithelial development as being essential for the development of rough Endoplasmic Reticulum (Beaulieu and Calvert, 1981). EGF has also been identified as a possible important regulatory molecule in lung epithelial cell differentiation (Sundell *et al.*, 1980; Gross *et al.,* 1986). Studies investigating fetal lung development showed localisation in the developing bronchi and around both Clara cells and type II pneumocytes (Raaberg *et al.,* 1992).

Insulin has also shown to be important in lung growth and differentiation, with receptors for insulin being demonstrated on whole fetal lung. Their number appears to increase during late gestation (Ulane *et al.*, 1982). One of the possible reasons for the importance of insulin is that glucose is a major substrate for the synthesis of the phospolipid, phosphatidylcholine, in type II pneumocytes. The receptors for insulin have been reported on type II pneumocytes from a number of sources (Sugahara *et al.*, 1987; Shapiro *et al.*, 1986).

1.5.1.3 Cell-Cell and Cell-Matrix Influences.

An important component in embryonic development is cell-cell interactions such as epithelial-mesenchymal and epithelial-epithelial. These interactions may be of a diffusable nature (Jessell and Melton, 1992) or through cell-cell adhesion molecules. Such interactions remain important in adult tissues e.g. skin (Fusenig, 1994).

Various studies have shown that soluble factors from lung fibroblasts can influence the growth and differentiation of lung epithelial cells *in vivo* e.g. the onset of pulmonary surfactant at birth (Smith and Fletcher, 1979). These studies have been confirmed by *in vitro* studies with cultured type II pneumocytes grown on fibroblast feeder layers producing surfactant and retaining differentiation characteristics such as morphology (Shannon *et al.*, 1987). Studies have shown that lung fibroblasts produce these soluble factors in response to glucocorticoid action. For example, the culture of A549 (which possesses features of type II pneumoctyes) with media from fibroblasts exposed to dexamethatasone (a synthetic hydrocortisone analogue) show an increase

in various markers of type II pulmonary cell differentiation e.g. pulmonary surfactant (McCormick *et al.*, 1995; Speirs *et al.*, 1991).

Similarly, interactions with the extracellular matrix play an important role in both development of the lung and its repair by determining cell fate and maintaining cell differentiation (Dobbs, 1990; Rannels and Rannels, 1989). For example, specific domains of the extracellular matrix are believed to determine the positions of type I and type II pneumocytes during lung growth and development, as well as during repair of the alveolar surface after injury (Lwebuga-Mukasa, 1991).

Investigations with isolated type II pneumocytes appear to support this theory. These studies show that fibronectin (a dimeric glycoprotein) promotes a loss of type II differentiation and an acquisition of type I characteristics, while laminin (a trimeric glycoprotein) promotes the retention of type II pneumocyte function (Rannels and Rannels, 1989; Rannels *et al.,* 1987). Furthermore, it appears that type II pneumocytes are responsible for the composition of the extracellular matrix (Dunsmore *et al.* 1995) and that the fibronectin-rich extracellular matrix (ECM) produced by isolated type II pneumocytes *in vitro* resembles the type I pneumocyte matrix (Rannels *et al.* 1987).

1.5.1.4 Retinoic acid.

Retinoic acid (RA), a derivative of vitamin-A, has been implicated in the control of both cellular proliferation and differentiation, and has been shown to effect over 200 different gene products (Chytil, 1992). For example, RA regulates elastin production by lung fibroblasts during alveolar septal formation (McGowan *et al.*, 1995).

Within the cytoplasm of the cell, RA binds to cytoplasmic retinoic acid-binding proteins (CRABPs) I and II and retinol binding proteins (CRBPs) I and II (Chytil and Ong, 1983). It is unclear if these proteins act in the storage of retinoids or in their metabolism (Yost *et al.*, 1988). The expression of CRABP I occurs in both fetal and adult lung, while CRABP II is found in fetal lung but is absent in adult lung (Chytil, 1992).

In the nucleus RA acts through two sets of transcription factors, the retinoic acid receptors (RAR) α, β, and γ, and the retinoid X receptors (RXR) α, β, and γ (Petkovich *et al.*, 1987; Yu *et al.*, 1991). RARs bind all-*trans*- and 9-*cis*-retinoic acid, while the RXRs selectively bind 9-*cis*-retionic acid (Leblanc and Stunnenberg, 1995). These receptors (RAR and RXR) recognise specific RA-responsive elements (RARE) within the promoter regulatory regions of several genes (McGowan *et al.*, 1995).

In order to exert their influence, the receptors heterodimerise to each other, e.g. RXR:RAR, or with other members of the nuclear receptor super-family (Leblanc and Stunnenberg, 1995). This heterodimerisation leads to an increase in ligand specificity and the number of target genes that can be activated or repressed. Thus, the differential expression and ligand-selective activation of the six retinoid proteins leads to cell type-specific expression of programs for development and growth control (Sporn *et al.*, 1994).

The α, β and γ subtypes of the receptors regulate the expression of specific genes by being expressed in a specific spatial and temporal manner (Mangelsdorf *et al.*, 1994). For example, in the developing mouse embryo RARα was detected throughout the lung, while RARβ was localised near bronchi and RARγ only appeared in the lung late in gestation (Dollé *et al.*, 1990).

1.5.2 SYNTHETIC AGENTS OF DIFFERENTIATION, 5-BROMO-2-DEOXYURIDINE.

Low levels of the thymidine analogue 5-Bromo-2'-Deoxyuridine (BrdU) have been shown to alter the differentiation status of different kinds of cells. This modulation of differentiation may be inhibitory e.g. myoblast cells (O'Neill and Stockdale, 1974) or stimulatory e.g. neuroblasoma cells (Ross *et al.*, 1995). Recent work in this laboratory has shown that BrdU induces differentiation in a poorly differentiated lung carcinoma cell line, DLKP (McBride *et al.*, 1999; Meleady and Clynes, manuscript submitted).

The exact mechanism by which BrdU exerts its differentiation-modulating effects is unclear but incorporation into DNA is seen as essential. This involves BrdU being converted to bromodeoxyuridine monophosphate, which competes with thymidine for incorporation into DNA (O'Neill and Stockdale, 1974). Experimental evidence for this hypothesis comes from a study by Keoffler *et al.* (1983) which showed that a thymidine kinase-deficient human myeloid cell line (HL-60) was unable to incorporate BrdU into its DNA and subsequently failed to respond to the ability of BrdU to modulate its differentiation status.

A number of models exist to explain the ability of BrdU to modulate differentiation:

Model 1:

This model envisages that BrdU induces chromosomal breakages. These breakages and the associated chromosomal aberrations can be associated with stepwise changes in the differentiation of a cell. These breakages are at specific points called fragile sites, 32 of which have been identified in murine chromosomes. It proposed that BrdU associates with these fragile sites which are known to be recombinogenic (Alexander *et al.*, 1992). Some corroboration for this model comes from a study by Schwartz and Snead (1982) which found that BrdU seemed to concentrate within repetitive DNA nucleotide sequences rather than randomly throughout the nuclear DNA. However, such selective incorporation would suit the other models also.

Model 2:

BrdU alters the affinity of DNA sequences for regulatory proteins. Studies on the *lac* operon with BrdU incorporated showed that the *lac* suppressor was bound with greater affinity (Lin and Riggs, 1972).

Model 3:

In this model BrdU has been found to exert its effects on differentiation by alteration of a key regulatory gene(s) that alters transcription of genes involved in differentiation (Arnold *et al.*, 1988; Rauth and Davidson, 1993). In BrdU inhibition of myoblast differentiation, such an alteration occurs with the down-regulation or complete inhibition of the key regulatory gene, MyoD1 (Tapscott *et al.*, 1989; Nanthakumar

and Henning, 1995). It could be envisaged that BrdU-induced alteration of differentiation in other tissues e.g. inhibition in mammary epithelial cells and pancreatic acinar cells, could be by a similar mechanism. This is strengthened by the homology of MyoD1 to the *myc* family of proteins, which have an important role in differentiation.

Indeed, in BrdU-induced differentiation of neuroblastoma, a decrease of both N-*myc* protein and mRNA levels occur (Ross *et al.*, 1995).

Model 4:

This model envisages that BrdU incorporation causes an alteration in the reading frame of the DNA template resulting in the formation of an abnormal mRNA, which is incapable of synthesising the correct differentiation products (Hill *et al.*, 1974).

1.6 CELL ADHESION MOLECULES.

1.6.1 INTRODUCTION.

The expression on the surface of cells of various types of cell adhesion molecules influences cell-cell sorting, tissue architecture and cellular differentiation. Cell adhesion molecules carry out these functions by binding other cell adhesion molecules or by binding to the extra-cellular matrix. The cell adhesion molecules are divided into several families such as integrins, selectins, cadherins and the immunogloblulin cell adhesion molecules. Alterations in the expression of these molecules have been linked to various pathological conditions, for example, the development of malignancy.

1.6.2 INTEGRINS.

The integrin receptors consist of two heterodimer chains, α and β, both of which form a non-covalently associated complex (Hynes, 1987). The α subunit family of integrins possesses 15 variants, while the β subunit family contains 8 variants. Thus in theory, the α and β subunits could associate to give over 100 integrins. However, the actual diversity is much more restricted and in reality the α and β subunits combine into 22 different integrins (Buck and Horwitz, 1987). The integrin family is sub-divided on the basis of its β subunit (Newham and Humphires, 1996). For example, the β_1 integrins are involved principally in the adhesion between the ECM and the cellular cytoskeletion (Buck *et al.*, 1987), while the β_2 integrins participate in cell-cell interactions (Ruoslahti, 1991). The specificity of binding is not determined solely by integrin pairing but also by the cell type it is expressed in, for example the $\alpha_2\beta_1$

integrin expressed on platelets will not bind laminin (Staatz *et al.*, 1989) while this integrin expressed on other cell types will bind laminin (Elices and Hemler, 1989).

Integrins have been implicated in such diverse processes as inflammation, cellular growth, differentiation, and cell polarity (Albelda and Buck, 1990). For example, the interaction in developing lung between the ECM and the epithelium is mediated by integrin receptors, and allows normal lung branching to occur (Gumbiner, 1996).

As well as functioning as cell adhesion molecules, the integrins have signalling functions that regulate various aspects of cell behaviour and differentiation. This signalling is accomplished through the focal adhesion proteins, some of which have intercellular signalling functions (LaFlamme *et al.*, 1992). In particular, the phosphorylation of focal adhesion kinase (FAK) can lead to a signalling event through the microtubial associated protein (MAP) kinase pathways (Davis, 1993).

1.6.3 CADHERINS.

There are at least twelve known members of the cadherin family, which are divided into subclasses, sharing a common basic structure. The three main subclasses are E-Cadherin (found on many types of epithelial cells), P-Cadherin (found in the placenta and epidermis), and N-Cadherin (found on nerve, heart and lens cells) (Takeichi, 1991).

The cadherins function as Ca^{2+}-dependent homophilic cell-cell binding proteins (Nose *et al.*, 1990). They are believed to modulate differentiation by co-signalling with other cell adhesion molecules e.g. E-cadherin and the integrins act together to modulate glandular differentiation in colorectal cells (Pignatelli *et al.*, 1992)

The cadherin molecules interact with the actin cytoskeleton via the catenins (α, β, and γ) located in the focal adhesion complexes (Ozawa *et al.*, 1990). Through these

interactions with the catenins to the cytoskeleton, the cadherins form cell junctions in epithelial cells, which are crucial for epithelial cell polarity (Ranscht, 1994). A disruption of these catenins leads to a disruption of cadherin function (Ozawa *et al.*, 1990). E-cadherin can signal downstream via β-catenin, which is an important component of the WNT-mediated signalling pathway (Christofori and Semb, 1999; Dale, 1998).

Epithelial-cadherin (E-Cadherin) is a glycoprotein with a molecular weight of 124kDa and is thought to be important during embryonic development (Shirayoshi *et al.*, 1986). It is also involved in generating and maintaining epithelial layers in adult tissues (Shirayoshi *et al.*, 1986).

Down-regulation of the E-cadherin/catenin complex has been implicated in oesophageal cancer (Kadowaki *et al.*, 1994), gastric cancer (Streit *et al.*, 1996) and colon cancer (Vermeulen *et al.*, 1995). E-cadherin suppression is also associated with various stages of differentiation and development (Christofori and Semb, 1999). Down-regulation of E-cadherin mediated cell-cell adhesion can occur via signalling from the Rho family of small GTPases (Tapon and Hall, 1997). Two members of this family, cdc42 and rac1, have been shown to down-regulate E-cadherin, by activating IQGAP1, which competes for α-catenin with β-catenin thereby inducing the disassociation of α-catenin from the E-cadherin cell adhesion complex (Kuroda *et al.*, 1998).

1.6.3 IMMUNOGLOBULIN SUPER FAMILY CELL ADHESION MOLECULES.

The immunoglobulin super family cell adhesion molecules (CAMs) are so identified because they possess one or more domains homologous to those found on immunoglobulins. The CAMs are divided into sub-families; the three most important being neural cell adhesion molecule (N-CAM) (Cunningham, 1995), intercellular

adhesion molecule (I-CAM) (Montefort *et al.*, 1993) and vascular cell adhesion molecule (V-CAM). They are single pass transmembrane proteins, and mediate cell-cell adhesion by Ca^{2+}-independent homophilic binding. Knockout experiments in mice have shown their importance in development. For example, deletion of N-CAM results in distortion of the nervous system (Tomasiewicz *et al.*, 1993). Furthermore, the promoter regions of the CAM gene sequences contain targets for the products of the developmental *Hox* and *Pax* genes (Cunningham, 1995).

1.7 Ep-CAM, A CELL-CELL ADHESION MOLECULE.

1.7.1 INTRODUCTION.

The various proteins involved in cell-cell adhesion and cell-matrix interactions also have roles in a number of other cellular and morphological processes. These include proliferation, differentiation, cellular locomotion, tissue organisation and regulation of other cell adhesion systems. One such protein is Ep-CAM, which is also known as epithelial specific antigen (ESA), 17-1A antigen, epithelial glycoprotein (EPG), GA733-2 antigen, KSA, EPG40, EPG2, CO17-1A antigen, KS1/4 antigen, and MOC 31 antigen. The exact function of this protein has yet to be clarified, but it appears to function as a homophilic, Ca^{2+}-independent intercellular adhesion molecule, capable of mediating cell aggregation, preventing cell scattering, and directing cell segregation (Litvinov *et al.*, 1997). Ep-CAM is found expressed in a polarised manner on the basolateral (and sometimes the basal) surface of the majority of simple cuboidal, columnar, pseudo-stratified columnar and transitional epithelia (Simon *et al.*, 1990; Litvinov *et al.*, 1994).

1.7.2 SEQUENCE AND STRUCTURE OF Ep-CAM.

The gene family identified as GA733 is composed (to date) of two highly homologous genes GA733-1 and GA733-2. Ep-CAM has been identified as the protein product of the latter (Linnenbach *et al.*, 1989; Szala *et al.*, 1990).

1.7.2.1 Sequence of Ep-CAM Gene.

The gene GA733-1 is an intronless functional gene. This unusual phenomenon is possibly due to retrotransposition (a flanking direct repeat is observed) and thus may be the mechanism for gene duplication in this family (with truncation occurring at the 319 untranslated sequence of the precursor cDNA) (Linnenbach *et al.*, 1989). Transcription of the GA733-1 gene produces a 1.8 kb. mRNA. The 35.7 kDa protein encoded is similar, but not identical to Ep-CAM (Szala *et al.*, 1990; Fornaro *et al.*, 1995).

The possible promoter region for GA733-1 includes a GC box, which appears to be identical to the simian virus GC, box IV though it is not clear yet if Ep-CAM is SP-1 responsive. In addition, present in the promoter region are an atypical CAAT box and a canonical TATA box (Linnenbach *et al.*, 1989).

The GA733-2 gene (Ep-CAM) is located on chromosome 4q (Helfrich *et al.*, 1997; Linnenbach *et al.*, 1993). When the sequence for Ep-CAM (GA733-2) was compared to the previously established cDNAs it emerged that GA733-2 consists of 9 exons interspaced with introns of variable length (Linnenbach *et al.*, 1989). Northern blot analysis has shown a single 1.5 kb mRNA species (Perez and Walker, 1989). cDNA studies show that the 3′-non coding region of Ep-CAM contains the sequence ATTTA. This sequence has been proposed as a recognition signal for mRNA initiation of cytokines and proto-oncogenes. The 3′-non coding region of Ep-CAM also contains a copy of TTATTTAT which has been identified as a consensus sequence in the 3′-non coding region of inflammatory mediators, suggesting that Ep-CAM shares a similar post-transcriptional regulatory mechanism with a number of proto-oncogenes and inflammatory mediators (Perez and Walker, 1989).

Limited northern blot data has indicated that GA733-1 and GA733-2 are expressed differently. High expression levels of GA733-1 mRNA are found in the pancreatic carcinoma cell line BXPC-3 relative to the colorectal cell line SW 948. Expression of GA733-2 (Ep-CAM) mRNA is low in the BXPC-3 cell line relative to the high expression level in SW 948 (Szala *et al.*, 1990). Such differences in expression levels

are not unanticipated since a characteristic feature of a retroposon, like GA733-1, is the acquisition of a heterologous promoter. In northern blots, no cross hybridisation between the two different-sized transcripts was observed, reflecting that at the DNA level the coding regions are only 54% identical (Szala *et al.*, 1990).

Also playing a role in the evolution of the Ep-CAM gene family is exon shuffling, as portions of these genes are found to be homologous to exon 8 of thyroglobulin. This exon shuffling would have preceded any gene duplication events of the GA733 family. The GA733 family also shows homology to exon 2 of the IL-2 receptor, which encodes for sequences involved in growth factor binding. The significance of the presence of these sequences is unknown (Linnenbach *et al.*, 1989). Ep-CAM is found to be highly conserved in a range of species. This is seen with highly homologous sequences to the human GA733-2 gene, which encodes for Ep-CAM, found in monkey, hamster, and chicken genomes, and an 85% homology between murine and human forms of Ep-CAM (Bergsagel *et al.*, 1992). This related family of conserved proteins are associated almost exclusively with epithelial tissues (Linnenbach *et al.*, 1993; Borkowski *et al.*, 1996).

1.7.2.2 Structure of Ep-CAM Protein.

Ep-CAM does not have any structural similarities to the other four major types of cell adhesion proteins, such as cadherins, integrins, selectins and the immunoglobulin superfamily (Litvinov *et al.*, 1997). It thus may represent a new family of cell surface proteins. The Ep-CAM protein is a 314 amino acid, 40 kDa type I (single pass) transmembrane glycoprotein with an isoelectric point between 6.9 and 7.7 (Ross *et al.*, 1986; Linnenbach *et al.*, 1989).

Over 80% of the mature protein is expressed extracellularly. This N-terminal extracellular domain contains two cystine-rich EGF-like domains followed by a cystine poor region. The two EGF-like repeats overlap a thyroglobulin-like repeat. It also has a small domain homologous to nidogen (an extracellular laminin binding protein) and placental protein 12 (an IGF-1 binding protein), though it does not share any functional similarities (Simon *et al.*, 1990; Litvinov *et al.*, 1994). The

hydrophobic transmembrane region contains 21 amino acids. On the cytoplasmic side, there is a basic 4 amino acid stop transfer sequence which is followed by a short (26 amino acid) cytoplasmic tail (Simon *et al.,* 1990; Litvinov *et al.,* 1997), of which 9 are positively charged (Linnenbach *et al.*, 1989). The extracellular region also contains three potential N-linked glycosylation sites (Asn-Xaa-Ser/Thr). Posttranslational glycosylation of these sites increases the molecular weight from 34 kDa for the *de novo* protein to its final weight of approximately 40 kDa (Simon *et al.*, 1990; Litvinov *et al.*, 1994).

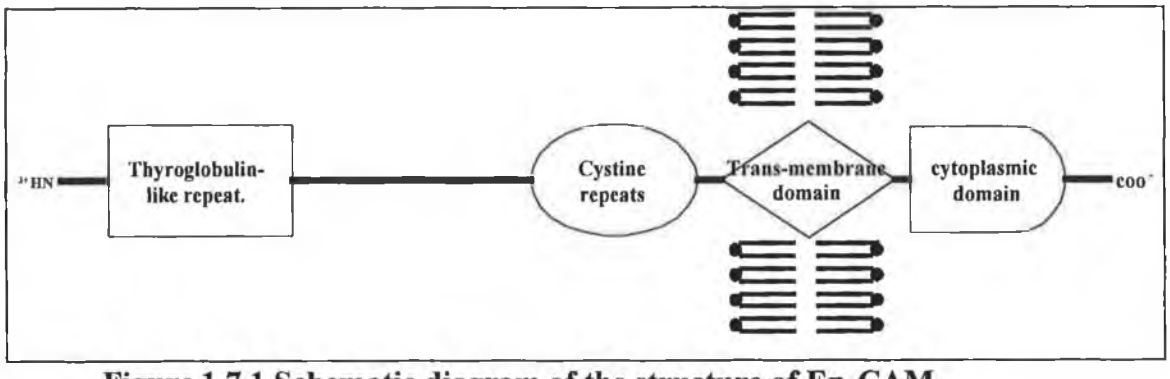


Figure 1.7.1 Schematic diagram of the structure of Ep-CAM.

Comparison of the amino acid sequences of GA 733-1 and GA 733-2 (Ep-CAM) with alignment programs shows that the two proteins are 49% identical. However, when conserved substitutions are taken into account this increases to 67% homology. Strong conservation occurs with the position of hydrophobic and hydrophilic residues in both antigens (Szala *et al.*, 1990).

There exists two regions of high homology, one is located in the transmembrane region with the proteins sharing 97% homology. The other region of high homology is in the extracellular domain. This 39 residue region is 79% homologous. The homologous extracellular domain is in turn homologus to the type I repeat of the thyrogloglobulin and (human leukocyte antigen-DR) HLA-DR associated invariant chains (Szala *et al.*, 1990).

1.7.3 FUNCTION AND MECHANISM OF Ep-CAM.

As stated earlier, Ep-CAM appears to be involved in cell-cell adhesion. Evidence for its role in cell-cell adhesion comes from *in vitro* studies which showed no involvement of Ep-CAM in cell-substrate contacts on a number of substrates, rather Ep-CAM was present at areas of intercellular contact (Litvinov *et al.*, 1994). Its exact functions have yet to be determined, but it may play a role in cell segregation in multilayered epithelia and in the organisation of epithelial tissues. Ep-CAM also is the main cell-cell adhesion molecule in most adenocarcinomas (Litvinov *et al.*, 1994). The location of Ep-CAM expressed on the membranes of highly differentiated cells is mainly in areas of intercellular contact. This differs from tumour cell where expression is homogenous on the cell membrane (Simon *et al.*, 1990; Litvinov *et al.*, 1994).

Ep-CAM has been implicated in cell-cell sorting in a number of tissues, in particular, glandular epithelia. Where two layers of epithelia are present Ep-CAM is not expressed in the basal layer. This differential expression may be important in sorting mature ductal and alveolar epithelial cells from a stem cell/myoepithelial cell population. Other organs where Ep-CAM cell segregation may play a function includes the pancreas and the sorting of β cells from non-β Islet cells, as β-islet cells are reported to express Ep-CAM (Litvinov *et al.*, 1994). In human fetal pancreas the highest levels of Ep-CAM expression occurs in developing β-islet-like cell clusters budding from the ductal epithelium, a cell compartment thought to comprise of endocrine progenitors. In the adult, the reverse pattern was observed with the β-islet cells exhibiting the lowest level of Ep-CAM and the ductal cells the highest. It was also shown that blockage of the Ep-CAM function by KS1/4 MAb induced insulin and glucagon gene transcription and translation in fetal pancreatic cell clusters (Cirulli *et al.*, 1998).

In vitro aggregation studies with Ep-CAM-transfected and non-transfected cells showed that they aggregated separately (Litviniov *et al.*, 1994). This can be explained in part by the finding that high Ep-CAM expression diminishes the effect of other

intercellular contacts (Litvinov *et al.*, 1994; Litvinov *et al.*, 1997). This feature is similar to that reported by Benchimol *et al.* (1989) for carcinoembryonic antigen.

These changes in intercellular adhesion are through alteration of cadherin-mediated cell-cell adhesion (Litvinov *et al.*, 1997). Transfection of Ep-CAM into E-cadherin (E-CAD) positive cells did not reduce the number of E-CAD molecules, but rather caused a redistribution of them on the cell surface and a reduction in the number of E-CAD mediated cell-cell adhesions (Litvinov *et al.*, 1997). E-CAD interacts with the actin filament component of the cytoskeleton via α- and β-catenins (figure 1.7.2(a)).

Investigation of these interactions in Ep-CAM-transfected cells revealed a possible mechanism of action (Litvinov *et al.*, 1997). In all cells (wild type and transfected), approximately similar levels of β-catenin was found irrespective of the level of Ep-CAM expression. There was however, a reduction in the β-catenin detergent insoluble fraction in transfected cells. Of more significance, was a reduction in both the total and detergent insoluble fractions of α-catenin (i.e. the fraction associated with the cell membrane focal adhesions). Thus, Ep-CAM expression effects E-CAD junctions by a reduction in α-catenin. Similar results were obtained in cells where the main cadherin is N-CAD, e.g. the human mammary cell line HBL-100. When the Ep-CAM levels are elevated in a cell, adhesion mediated by E-CAD and N-CAD is weakened and replaced with Ep-CAM intercellular adhesion, suggesting a co-ordination between the molecules rather then a simple anti-adhesion effect (Litvinov *et al.*, 1997).

Induction of high levels of Ep-CAM also changes morphology and leads to a more scattered phenotype, although the cells still remain attached to each other. Cells transfected with a mutant Ep-CAM molecule, which was lacking the cytoplasmic domain showed no alteration to E-CAD distribution and binding (Litvinov *et al.*, 1997). This result implies that the cytoplasmic domain of Ep-CAM is important for the co-ordinated regulation of the two molecules. Ep-CAM interacts with the actin-based cytoskeleton via α-actinin without the involvement of either α- or β-catenin (figure 1.7.2.(b)) (Balzar *et al.*, 1998). Ep-CAM appears to possess two potential binding sites for α-actinin on its cytoplamic tail at positions 289 to 269 and 304 to 314 (Balzar *et al.*, 1998). This suggests that Ep-CAMs negative effect on cadherin

junctions may be due to a redistribution of α-actinin molecules away from cadherin molecules to Ep-CAM. An alternative mechanism may be Ep-CAM actively signalling which leads ultimately to a down-regulation of α-catenin, This mechanism for action exists for ICAM-1 which down-regulates β_1 integrin expression to which Ep-CAM possess some similarities (Litvinov *et al.*, 1997).

E-cadherin is capable of down-regulating β_1 integrin expression during terminal differentiation in keratinocytes (Hodivala and Watt, 1994). Thus if Ep-CAM alters the function of E-cadherin an increase in β_1-integrin should occur. Such a relationship can be observed in cervical intraepithelial neoplasia lesions where the expression of β_1 integrin is similar to that of Ep-CAM (Litvinov *et al.*, 1996).

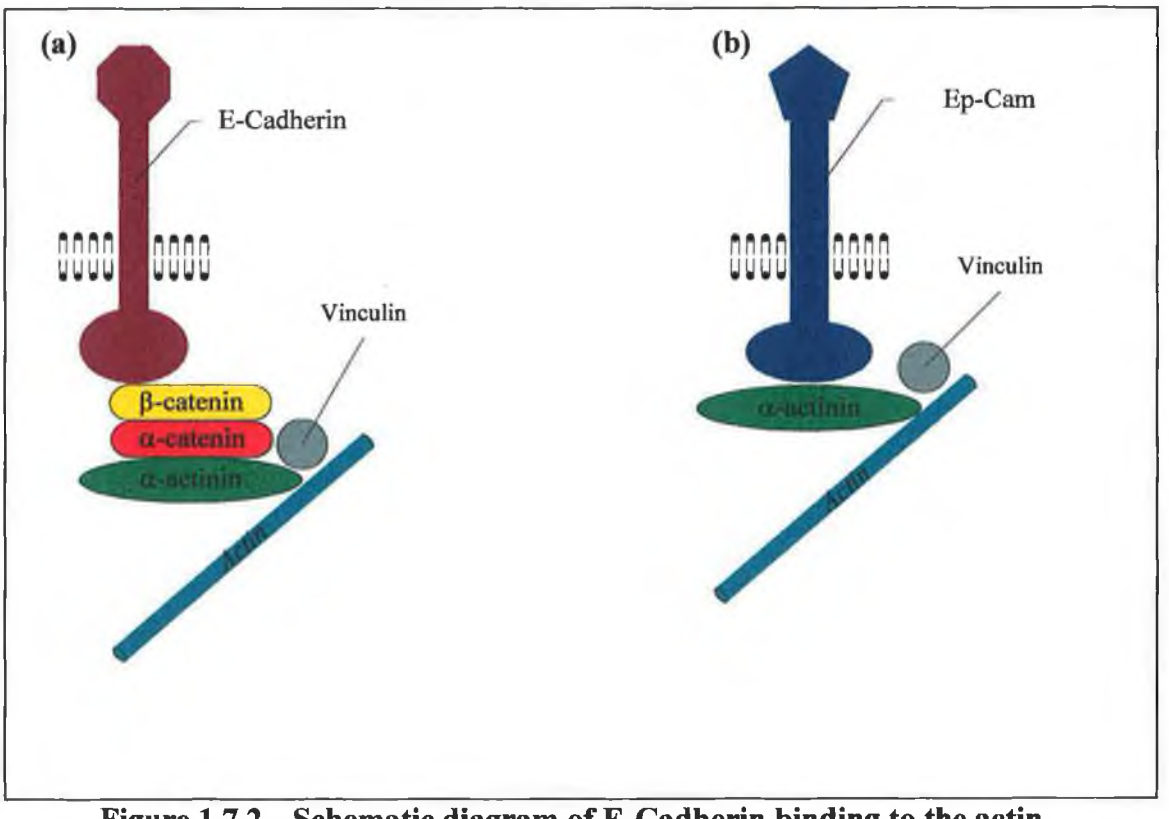


Figure 1.7.2 Schematic diagram of E-Cadherin binding to the actin cytoskeleton (A). Schematic diagram of Ep-CAM binding to the actin cytoskeleton (B). Note that in both cases a number of other proteins may also be involved to form focal adhesions e.g. Vinculin.

An alternative function for the GA733 family has been suggested by study by Fornaro *et al.* (1995). They found that both GA733-1 and GA733-2 had homology to IGF-II

binding proteins and could find no role for the proteins in cell adhesion. Ripani *et al.* (1998) found that the cytoplasmic tail of GA733-1 possesses potential serine and tyrosine phosphorylation sites and a phosphatidyl-inositol binding consensus sequence. Using antibodies against the protein, increased internal calcium levels were observed in MCF-7 (breast) and OvCA-432 (ovarian) cell lines. Thus, the alternative proposed function for the GA733 family is that of a cell receptor for a yet unidentified ligand.

1.7.4 REGULATION OF Ep-CAM EXPRESSION.

The regulation of Ep-CAM expression is still not fully elucidated. However, it does appear that cell density is important. In a series of experiments, Litivinov *et al.* (1994) observed that in cultures where single cells predominated (5% confluency), Ep-CAM was present at the pseudo-apical domains of the cell membrane. In cultures of greater density (70% confluency), the majority of Ep-CAM molecules relocated to the cell-cell boundaries. The formation of intercellular contacts seems to cause a slight decrease in Ep-CAM at the surface, as shown by flow cytometry. Low calcium caused a slight decrease in surface expression in the differentiated cell lines RC-6 and MCF-7 (Litivinov *et al.*, 1994).

1.7.5 TISSUE DISTRIBUTION OF Ep-CAM.

Ep-CAM has a wide distribution in normal human epithelia, with expression to be found on most simple, columnar and pseudostratified epithelia e.g. bronchiolar and alveolar epithelial cells, mucous acinar cells in the gastrointestinal tract, pancreatic islet cells and thyroid follicular cells (Momberg *et al.*, 1987). No Ep-CAM expression occurs in squamous stratified epithelial cells, epidermal keratinocytes, gastric parietal

cells, fibroblasts, thymic cortical epithelium, myoepithelial cells, hepatocytes and the peripheral blood of bone marrow (Momberg *et al.*, 1987; Moldenhauer *et al.*, 1987; Pantel *et al.*, 1993; Litvinov *et al.*, 1994; Litvinov *et al.*, 1996). As no expression of Ep-CAM is detected in peripheral blood of bone marrow with MAbs, it is considered a useful tumour marker for circulating tumour cells and bone marrow development (Momberg *et al.*, 1987).

In most adenocarcinomas increased levels of Ep-CAM can be found (Varki *et al.*, 1984), and in carcinomas that originate from squamous epithelia, *de novo* expression of Ep-CAM can be observed (Litvinov *et al.*, 1996). Particularly strong staining reactions are usually seen in colon carcinoma and small cell lung carcinoma cell lines (Moldenhauer *et al.*, 1987). Ep-CAM is believed to function as the main intercellular adhesion protein for some carcinoma cells (Litvinov *et al.*, 1994). The distribution of Ep-CAM on the cell membrane of the breast cell line RC-6 is different in single cells as compared to colonies. In single cells the molecule is distributed more widely to include the pseudoapical domain, but when two cells form a stable intercellular contact, the Ep-CAM molecules relocate to the lateral domains (Litvinov *et al.*, 1994). The membrane distribution of Ep-CAM differs between normal cells and tumour cells, with normal cells exhibiting an apolarised expression while tumour cells show a more homogenous staining (Simon *et al.*, 1990). For example, in normal glandular tissues e.g. mammary gland epithelium Ep-CAM is found localised mainly at cell-cell boundaries between epithelial cells. In some mammary carcinomas the intensity of staining may be less then in normal tissue with some showing an increase in cytoplasmic staining compared to membrane staining (Litvinov *et al.*, 1994). In a study by Moldenhauer *et al.* (1987) using the anti- Ep-CAM antibody HEA125 against a panel of human cell lines, no reaction was observed in any of the non-carcinoma cell lines used, which were derived from melanoma, neuroblastoma, sarcoma and leukaemia/lymphoma.

Litvinov *et al.* (1996) investigated the expression of Ep-CAM in cervical squamous epithelia. It was noted that reserve cells, which are capable of differentiating into squamous epithelia both *in vitro* and in nude mice, expressed Ep-CAM. This normal expression of Ep-CAM is repressed as soon as the trans-differentiating cells acquired the squamous phenotype. However, in dysplastic/neoplastic squamous epithelium this

expression continues. The survey also showed that the appearance of Ep-CAM in cervical squamous epithelia correlated with loss of tissue-specific markers, including the markers for terminal differentiation of squamous epithelial cells and expression of markers for simple epithelia and enhanced proliferative activity. Cytokeratin 13 (a squamous differentiation related cytokeratin) expression was observed mainly in Ep-CAM negative regions of mature squamous metaplasia. Ep-CAM positive cells also failed to express involucrin (a marker of terminal differentiation of keratinocytes).

The expression patterns of simple epithelial cell cytokeratins 8 and 18 and of Ep-CAM were identical in metaplastic tissue of the utrine cervix (Litvinov *et al.*, 1996). The expression of Ki-67, a marker for proliferation, was found to be concurrent with Ep-CAM expression. Co-expression also occurred with cytokeratins 5 and 14, which mark proliferating cell populations. The expression of Ep-CAM in squamous cervix epithelia is clearly a disturbance of normal proliferation and differentiation, and reflects an early event in cervical carcinogenesis. Hence, Ep-CAM may serve as an early marker of dysplastic/neoplastic changes in cervical squamous epithelium.

1.7.6 CLINICAL RELEVANCE OF Ep-CAM.

Ep-CAM is of clinical interest as a prognostic indicator (Varki *et al.*, 1984; Songun *et al.*, 1996), as a means of detecting metastatic cells in peripheral blood, microtumour visualisation via radioimmunolocalization (Momburg *et al.*, 1987; Kievit *at al.*, 1997) and as a possible target of various therapies.

Clinical interest exists in the detection of circulating tumour cells in peripheral blood as such detection may aid in the choice of treatment given to a patient. For example, in treatments involving the use of intensive high-dose chemotherapy combined with autologous bone marrow transplantation or peripheral stem cell reinfusion, the presence of contaminating tumour cells may worsen the prognosis (Helfrich *et al.*, 1997; de Graaf *et al.*, 1997). As Ep-CAM has not been found with monoclonal antibodies on peripheral blood cells or bone marrow (Moldenhauer *et al.*, 1987) to this

end, RT-PCR of Ep-CAM has been investigated as a possible highly sensitive and reliable method of detection and quantification of circulating tumour cells (Helfrich *et al.*, 1997; de Graaf *et al.*, 1997). Both labs found that this method was not reliable enough to use in a clinical situation. The potential problems included low level illegitimate (ectopic) expression of Ep-CAM in bone marrow and peripheral blood cells. The significance of this illegitimate expression is not known, but similar expression has been found for cytokeratin 19 (Krissmann *et al.*, 1995) and prostate-specific antigen (Smith *et al.*, 1995) and PGP-9.5 (Norris *et al.*, 1994).

It was also found that among a panel of cell lines, widely different levels of expression was detected with cell lines such as MDA-MB431 (breast carcinoma) and GLC4 (small-cell lung carcinoma) showing very low levels of expression and cell lines such as MCF-7 (breast carcinoma) and T47D (breast carcinoma) showing very high levels of expression. Thus, quantification of the number of tumour cells present was not possible. In spiking experiments, de Graaf *et al.* (1997) could detect only one positive cell per 2 x 10^4 mononucleucocytes where as Helfrich *et al.* (1997) could detect one positive cell per 1 x 10^5 mononucleucocytes. Monoclonal antibody techniques can usually detect one carcinoma cell per $1x10^4$ - 5 x 10^5 mononucleucocytes (de Graaf *et al.* 1997; Helfrich *et al.* 1997). Thus, further refinement is needed before RT-PCR is suited to a clinical setting. However, it may prove to be more reliable when used in conjunction with monoclonal antibody methods.

Due to its high expression in tumour cells, Ep-CAM has been tested for its ability to induce tumour kill by cytotoxic T lymphocytes. In a study by Ras *et al.* (1997) 410 peptides derived from Ep-CAM were screened by their binding characteristics to the human leukocyte antigen, HLA-A*0201. From this, six peptides were selected for further studies. HLA-A*0210K^b transgenic mice were immunised with these peptides and the T lymphocytes were isolated from the spleens and tested for their ability to lyse Jurkat HLA-A*0210K^b cells exposed to peptide. Only one peptide, with the sequence GLKAGVIAV, showed a response. In a different approach, Kroesen *et al.* (1997) developed a chimeric MAb (called BIS-1) which has specificity for the CD3 complex present on all T lymphocytes and is specific for Ep-CAM. The T-cells were pre-activated *in vitro* prior to treatment of the carcinoma patients involved in the study

(who suffered from malignant ascites or interpleural effusions). Though the *in vitro* assays with the patients blood demonstrated anti-tumour activity and previous studies with rat models showed that BIS-1/IL-2 could be effective against low systemic tumour burdens, no clear clinical responses were observed in the patients (Kroesen *et al.*, 1997).

Ep-CAM is of interest to those investigating the use of MAb conjugates for the treatment of various malignancies. In one such study, Zimmerman *et al.* (1997) combined the MAb MOC31 with a recombinant form of Pseudomonas exotoxin A, which lacks the cell-binding domain ETA252-613. This construct, at concentrations of 0.01nM to 0.3nM, gave a 50% inhibition in the growth of the small cell lung carcinoma (SCLC) and adenocarcinoma cell lines tested. In athymic mice, regression was seen in small (40 mm^3) chemoresistant tumour xenografts. In larger tumours (120 mm^3), growth was delayed. In another study, Elias *et al.* (1990) evaluated a KS1/4-methotrexate immunoconjugate in patients with stage IIIB or IV NSCLC. Patients received a dose maximum of 1661 mg. The study yielded just one possible clinical response, which reveals the problems associated with MAb conjugated to chemotherapy agents, toxins, or radioisotopes infused into patients. As most monoclonal antibodies utilised are murine, treatment often elicits a human anti-murine antibody response. Such responses can cause IgE-mediated allergic reactions or even more serious type 3 immune responses with associated organ and/or tissue damage. Such reactions may also effect MAb distribution and may neutralise it or prevent adequate tumour binding. In a study looking at this anti-murine antibody response, Petersen *et al.* (1991) investigated two KS1/4 *Vinca* alkaloid conjugates KS1/4-desacetylvinblastine and KS1/4-desacetyl-vinblastine-3-carboxyhydrazide. In the 44 patients treated, 32 elicited an anti-mouse antibody response.

1.8 AIMS OF THESIS

Previous research in this laboratory has shown that the halogenated thymidine analogue, bromodeoxyuridine (BrdU) induces the *in vitro* differentiation of the lung cell lines DLKP and A549. This differentiation is indicated by induction of the early cytokeratins –8, -18 and –19 (McBride *et al.*, 1999; Meleady and Clynes, manuscript submitted). Also shown to be induced by BrdU are the integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ (Paula Meleady, Ph.D thesis 1997, DCU). Preliminary research by Dr. Meleady indicated that the cell-cell adhesion molecule Ep-CAM was also induced in these cell lines following BrdU treatment.

1.8.1 THE ROLE OF EP-CAM IN EPITHELIAL LUNG CELL LINE DIFFERENTIATION.

- The main aim of this thesis to confirm, and expand on this preliminary result. Knowledge of the role and function of Ep-CAM in the differentiation of epithelial lung cells is very limited. It was the aim of this work to use the induction of differentiation in DLKP and A549 as models for the role of Ep-CAM in lung differentiation. In this thesis, the molecular mechanism by which BrdU modulates Ep-CAM induction was to be addressed. Such information may aid in the elucidation of the control of expression of Ep-CAM *in vivo*.
- It is believed that Ep-CAM may cause an alteration in the levels of focal adhesion proteins, thereby modulating the function of other cell adhesion proteins; it may therefore have a role in cell-cell sorting. Another aim of the work conducted in this thesis was to investigate the effect of Ep-CAM induction on two focal adhesion proteins, utilising BrdU-treated DLKP and A549 as models.
- It was also decided to perform preliminary research on the effect of BrdU on the translation of GA733-1 (a homologue of Ep-CAM). There is no literature available on the role of GA733-1 in differentiation, and only limited studies have been performed on its expression *in vivo*.

1.8.2 THE EFFECT OF OTHER HALOGENATED THYMIDINE ANALOGUES ON EPITHELIAL LUNG CELL DIFFERENTIATION.

- The halogenated thymidine analogue BrdU has been shown in this laboratory to induce the expression of cytokeratin and integrin proteins. The ability of other halogenated thymidine analogues to alter the expression of these proteins was also to be investigated.
- It was hoped that such an investigation would help us gain a better understanding of the mechanisms by which differentiation may be regulated. Potentially, this knowledge could be used in the design of therapeutics for differentiation therapy.

1.8.3 THE DEVELOPMENT OF *IN VITRO* MODELS THAT REFLECTED *IN VIVO* DIFFERENTIATION.

- As part of our programme to design *in vitro* models of *in vivo* differentiation, we decided to build on the research of Emura (1997). Who described a complex hormone supplemented media that induced differentiation in hamster fetal epithelial lung cell line. Work presented in this thesis investigated if various modifications of the medium induced differentiation in the lung epithelial cell lines DLKP and A549. Further investigations were conducted to identify which were most significant modulators of differentiation, by the deletion of specific components of this media.
- Investigations were conducted into the development of *in vitro* models that reflected *in vivo* differentiation. This involved the establishment of primary cultures of type II pneumocytes to represent normal cells and primary cultures of lung tumours to represent cancer states. In the course of this study, fibroblast cells were isolated; unusually these were found to be cytokeratin positive. Preliminary investigations were conducted into the expression of cytokeratin, by the fibroblast cells derived in the course of this thesis and by three primary cultures of fibroblasts sourced from other established culture collections.

2.0 MATERIALS AND METHODS.

2.1 WATER

Ultrapure water was used in the preparation of all media and solutions. This water was purified by a reverse osmosis system (Millipore Milli-RO 10 Plus, Elgastat UHP) to a standard of 12 - 18 MΩ/cm resistance.

2.2 GLASSWARE

All solutions for to cell culture and maintenance were prepared and stored in sterile glass bottles. Bottles (and lids) and all other glassware used for any cell-related work was prepared as follows: - all glassware and lids were soaked in a 2% (v/v) solution of RBS-25 (AGB Scientific) for at least 1-hour. This is a deproteinising agent, which removes proteineous material from the bottles. Glassware was scrubbed and rinsed several times in tap water, the bottles were then washed by machine using Neodisher detergent, an organic, phosphate-based acid detergent. The bottles were then rinsed twice with distilled water, once with ultrapure water and sterilised by autoclaving.

2.3 STERILISATION

Water, glassware and all thermostable solutions were sterilised by autoclaving at 121°C for 20 minutes (min) under pressure of 1bar. Thermolabile solutions were filtered through a 0.22μm sterile filter (Millipore, millex-gv, SLGV-025BS). Low protein-binding filters were used for all protein-containing solutions.

2.4 MEDIA PREPARATION

Medium was routinely prepared and sterility checked by Mr. Joe Carey (technician) as in SOP NCTCC 003-02. The basal media used during routine cell culture were prepared according to the formulations shown in Table 2.4.1. 10x media were added to sterile ultrapure water, buffered with HEPES and $NaHCO_3$ and adjusted to a pH of 7.45 - 7.55 using sterile 1.5M NaOH and 1.5M HCl. The media were then filtered through sterile 0.22μm bell filters (Gelman, 121-58) and stored in 500ml sterile bottles at 4°C. Sterility checks were carried out on each 500ml bottle of medium as described in Section 2.5.5.

The basal media were stored at 4°C up to their expiry dates as specified on each individual 10x medium container. Working stocks of culture media was prepared as 100ml aliquots, supplemented with 2mM L-glutamine (Gibco, 25030-024) and 5% fetal calf serum (Bio-Whittaker; 14-601F, Lot No.- 55B007; Sigma, F7524, Lot No.- 48H3377). This was stored for up to 2 weeks at 4°C, after which time, fresh culture medium was prepared.

	DMEM (Gibco, 12501-029)	Hams F12 (Gibco, 21700-109)	MEM (Gibco, 21430-020)
10X Medium	500ml	Powder	500ml
Ultrapure H_20	4300ml	4700ml	4300ml
1M HEPES* Sigma , H-9136	100ml	100ml	100ml
7.5% $NaHCO_3$ BDH, 30151	45ml	45ml	45ml

* HEPES = N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)

Table 2.4.1 Preparation of basal media

For most cell lines, ATCC (Ham's F12/ DMEM (1:1)) supplemented with 5% FCS and 2mM L-glutamine was routinely used. MEM was supplemented with 5% FCS, 2mM L-glutamine, 1x NEAA (Gibco, 11140-035) and 1x sodium pyruvate (Gibco, 11360-039).

2.5 CELL LINES

All cell culture work was carried out in a class II down-flow re-circulating laminar flow cabinet (Nuaire Biological Cabinet) and any work, which involved toxic compounds, was carried out in a cytoguard (Gelman). Strict aseptic techniques were adhered to at all times. The laminar flow cabinet was swabbed with 70% industrial methylated spirits (IMS) before and after use, as were all items used in the cabinet. Each cell line was assigned specific media and waste bottles and only one cell line was worked with at a time in the cabinet which was allowed to clear for 15min between different cell lines. The cabinet itself was cleaned each week with industrial

detergents (Virkon, Antec. International; TEGO, TH.Goldschmidt Ltd.), as were the incubators.

The cell lines used during the course of this study, their sources and their basal media requirements are listed in Table 2.5.1. Lines were maintained in 25cm^2 flasks (Costar; 3050) or 75cm^2 flasks (Costar; 3075) at 37°C and fed every two to three days.

2.5.1 SUBCULTURE OF ADHERENT LINES

During routine sub-culturing or harvesting of adherent lines, cells were removed from their flasks by enzymatic detachment.

Waste medium was removed from the flasks and rinsed with a pre-warmed (37°C) trypsin/EDTA (TV) solution (0.25% trypsin (Gibco, 25090-028), 0.01% EDTA (Sigma, EDS) solution in PBS A (Oxoid, BR14a)). The purpose of this was to inhibit any naturally occurring trypsin inhibitor, which would be present in residual serum. Fresh TV was then placed on the cells (2ml/25cm^2 flask or 4ml/75cm^2 flask) and the flasks incubated at 37°C until the cells were seen to have detached (5-10 min). The trypsin was deactivated by addition of an equal volume of growth medium (*i.e.* containing 5% serum). The entire solution was transferred to a 30ml sterile universal tube (Sterilin; 128a) and centrifuged at 1,000 rpm for 5 min. The resulting cell pellet was resuspended in pre-warmed (37°C) fresh growth medium, counted (Section 2.5.3) and used to re-seed a flask at the required cell density or to set up an assay.

Cell Line	Cell Type	Source	Basal Medium
DLKP	Human poorly-differentiated lung carcinoma	Dr. Geraldine Grant, NCTCC	Hams F-12:DME (1:1) (ATCC).
A549	Human lung adenocarcinoma	ATCC American Type Culture Collection	Hams F-12:DME (1:1) (ATCC).
MCF-7	Human breast carcinoma	ATCC American Type Culture Collection	MEM

Table 2.5.1 Cell lines used during the course of study.

2.5.3 CELL COUNTING

Cell counting and viability determinations were carried out using a trypan-blue (Gibco, 15250-012) dye exclusion technique.

An aliquot of trypan-blue was added to a sample from a single cell suspension in a ratio of 1:5. After 3 min incubation at room temperature, a sample of this mixture was applied to the chamber of a haemocytometer over which a glass coverslip had been placed. Cells in the 16 squares of the four outer corner grids of the chamber were counted microscopically, an average per corner grid was calculated with the dilution factor being taken into account and final cell numbers were multiplied by 10^4 to determine the number of cells per ml (volume occupied by sample in chamber is 0.1cm x 0.1cm x 0.01cm *i.e.* $0.0001cm^3$ therefore cell number x 10^4 is equivalent to cells per ml). Non-viable cells were those, which stained blue while viable cells excluded the trypan-blue dye and remained unstained.

2.5.4 CELL FREEZING

To allow long term storage of cell stocks, cells were frozen and cryo-preserved in liquid nitrogen at temperatures below –180°C. Once frozen properly, such stocks should last indefinitely.

Cells to be frozen were harvested in the log phase of growth (*i.e.* actively growing and approximately 50-70% confluent) and counted as described in Sections 2.5.3. Pelleted cells were re-suspended in serum and an equal volume of a DMSO/serum (1:9, v/v). The solution was slowly added drop-wise to the cell suspension, as DMSO is toxic to cells. A final concentration of at least $5x10^6$ cells/ml was generated. The suspension was then aliquoted into cryovials (Greiner, 122 278) which were then quickly placed in the vapour phase of liquid nitrogen containers (approximately -80°C). After 2.5 to 3.5 hours, the cryovials were lowered down into the liquid nitrogen where they were stored until required.

2.5.5 CELL THAWING

Immediately prior to the removal of a cryovial from the liquid nitrogen stores for thawing, a sterile universal tube containing growth medium was prepared for the rapid transfer and dilution of thawed cells to reduce their exposure time to the DMSO freezing solution which is toxic at room temperature. The suspension was centrifuged at 1,000 rpm. for 5 min, the DMSO-containing supernatant removed and the pellet re-suspended in fresh growth medium. A viability count was carried out (Section 2.5.3) to determine the efficacy of the freezing/ thawing procedures. Thawed cells were placed into tissue culture flasks with the appropriate volume of medium (10ml/25cm^2 flask and 15ml/75cm^2 flask) and allowed to attach overnight. After 24 hours, the cells were re-fed with fresh medium to remove any residual traces of DMSO.

2.5.6 STERILITY CHECKS

Sterility checks were routinely carried out on all media, supplements and trypsin used for cell culture. Samples of basal media were inoculated into Columbia (Oxoid, CM331) blood agar plates, Sabauraud (Oxoid, CM217) dextrose and Thioglycollate (Oxoid, CM173) broth's which should between them detect most contaminants including bacteria, fungus and yeast. Growth media (*i.e.* supplemented with serum and L-glutamine) were sterility checked at least 2 days prior to use by incubating samples at 37°C which were subsequently examined for turbidity and other indications of contamination.

2.6 *MYCOPLASMA* ANALYSIS

Mycoplasma examinations were carried out routinely (at least every 3 months) on all cell lines used in this study. These analyses were performed by Dr. Mary Heenan, Ms. Bojana Cumpf, and Mr. Michael Henry.

2.6.1 INDIRECT STAINING PROCEDURE

In this procedure, *Mycoplasma*-negative NRK cells (a normal rat kidney fibroblast line) were used as indicator cells. As such, these cells were incubated with supernatant from test cell lines and then examined for *Mycoplasma* contamination. NRK cells were used for this procedure because cell integrity is well maintained during fixation. A fluorescent Hoechst stain was utilised which binds specifically to DNA and so will stain the nucleus of the cell in addition to any *Mycoplasma* DNA present. A *Mycoplasma* infection would thus be seen as small fluorescent bodies in the cytoplasm of the NRK cells and sometimes outside the cells.

NRK cells were seeded onto sterile coverslips in sterile Petri dishes at a cell density of $2x10^3$ cells per ml and allowed to attach overnight at 37°C in a 5% CO_2, humidified incubator. 1ml of cell-free (cleared by centrifugation at 1,000 rpm for 5 min) supernatant from each test cell line was then inoculated onto a NRK Petri dish and incubated as before until the cells reached 20 - 50% confluency (4-5 days). After this time, the waste medium was removed from the Petri dishes, the coverslips washed twice with sterile PBS A, once with a cold PBS/Carnoys (50/50) solution and fixed with 2ml of Carnoys solution (acetic acid:methanol-1:3)for 10 min. The fixative was then removed and after air drying, the coverslips were washed twice in deionised water and stained with 2ml of Hoechst 33258 stain (BDH)(50ng/ml) for 10 min.

From this point on, work proceeded in the dark to limit quenching of the fluorescent stain.

The coverslips were rinsed three times in PBS. They were then mounted in 50% (v/v) glycerol in 0.05M citric acid and 0.1M disodium phosphate and examined using a fluorescent microscope with a UV filter.

2.6.2 DIRECT STAINING

The direct stain for *Mycoplasma* involved a culture method where test samples were inoculated onto an enriched *Mycoplasma* culture broth (Oxoid, CM403) - supplemented with 16% serum. 0.002% DNA (BDH; 42026), 2mg/ml fungizone (Gibco,15290-026), $2x10^3$ units penicillin (Sigma, Pen-3) and 10ml of a 25% (w/v) yeast extract solution - to optimise growth of any contaminants and incubated at 37°C for 48 hours. Sample of this broth were then streaked onto plates of *Mycoplasma* agar base (Oxoid, CM401) which had also been supplemented as above and the plates were incubated for 3 weeks at 37°C in a CO_2 environment. The plates were viewed microscopically at least every 7 days and the appearance of small, "fried egg" -shaped colonies would be indicative of a mycoplasma infection.

2.7 DIFFERENTIATION STUDIES

2.7.1 HALOGENATED THYMIDINE ANALOGUES.

Differentiation studies were conducted using various halogenated thymidine analogues. The preparation, source, and final concentration of usage are summarised in table 2.7.1. All compounds were stored at –20°C

Compound	Source and Cat No.	Stock Concentration	Treatment Concentration
5-Bromo-2'-DeoxyUridine (BrdU)	Sigma, B5002	10mM in sterile UHP, and filter sterilise.	10μM
5-Fluro-5'-DeoxyUridine (5,5'-5,5'-FdU)	Sigma F8791	10mM in sterile UHP, and filter sterilise.	2μM
5-Chloro-2'-DeoxyUridine (CdU)	Sigma, C6891	10mM in sterile UHP, and filter sterilise.	10μM
5-BromoUridine (5-BUr)	Sigma, B9752	10mM in sterile UHP, and filter sterilise.	70μM

Table 2.7.1 Halogenated thymidine anloges used in differentiation studies.

2.7.2 HORMONE SUPPLEMENTED MEDIUM.

Differentiation studies were conducted using a hormone supplemented medium (HSM). To ATCC supplemented with 5% FCS, and 2mM L-glutamine was added the components in the concentrations stated in table 2.7.2. This was prepared on the day of use.

Component	Source and Cat. No.	Final Concentration.
Insulin	Sigma; I 1882	8μg/ml
Hydrocortisone	Sigma; H 0135	3μg/ml
Oestrogen	Sigma; E 2257	2.7ηg/ml
Epidermal Growth Factor (EGF)	Sigma; E 1264	20ηg/ml
Cholera Toxin	Sigma; C 8052	0.25μg/ml

Table 2.7.2 Components used for the preparation of HSM.

2.7.3 DIFFERENTIATION ASSAYS. IMMUNOCYTOCHEMISTRY.

2.7.3.1 Immunocytochemistry. Preparation of sample.

In all experiments, cells were plated onto 6-well plates (Costar, 3516) at densities of $5x10^3$ per well for DLKP and A549 1.5ml of medium was sufficient for each well. The cells were allowed to attach and form colonies by incubating at 37^0C, 5% CO_2 for 48 hours. The plates were covered with parafilm to prevent contamination. The medium was then removed and replaced with medium with the required concentrations of halogenated thymidine analogue, or with HSM. Plates were wrapped in aluminium foil because of the light-sensitive nature of some of the compounds being used e.g. oestrogen. The plates were incubated for up to 7 days. Medium was replaced every 2-3 days over the course of the assay. All waste medium

was retained for disposal by incineration.

At the end of the assay, medium was removed from the cells, the plates were rinsed 3 times with PBS A and the cells were fixed with methanol as described in Section 2.7.3.2. Immunocytochemistry was the carried out using a range of antibodies as described in Section 2.7.3.3.

2.7.3.2 Immunocytochemistry. Fixation of cells.

This procedure was used for all cells whether they were grown on 6-well plates, multiwell slides, or cytospins had been prepared from them. For fixation, cells were rinsed 3 times with PBS A and then incubated at -20^{0}C for 7 minutes using ice-cold methanol. The methanol was the removed from the cells and the cells were allowed to air-dry for a number of hours or overnight and then stored at -20^{0}C until required. This method appeared to be successful for all antibodies investigated during the course of the study.

2.7.3.3 Immunocytochemistry. Procedure.

The avidin-biotin complex (ABC) immunoperoxidase technique combined with the diaminobenzidine (DAB) visualisation procedure was used in all immunocytochemistry experiments. The ABC method involves application of a biotin-labelled secondary antibody to cells probed with a primary antibody, followed by the addition of avidin-biotin-peroxidase complex which results in a high staining intensity due to the formation of an avidin-biotin lattice which contains peroxidase molecules. The peroxidase enzyme then reacts with a DAB solution to give an insoluble, brown-coloured precipitate. The formation of this brown precipitate-coloured precipitate is indicative of primary antibody reactivity.

The procedure used is as follows:
Cell preparations (cytospins, multiwell slides, 6-well tissue culture plates) which had been previously fixed in methanol and frozen at -20^{0}C were allowed to thaw and equilibrate at room temperature. A grease pen (DAKO, S2002) was used to encircle

cells on cytospins, multiwell slides and in tissue culture plates to contain the various solutions involved. The cells were incubated for 5 minutes with a 3% H_2O_2 solution to quench any endogenous peroxidase activity that may be present in the cells and could lead to false positive results. The cells were then rinsed with UHP water and placed in Tris-buffered saline (TBS) (0.05M Tris/HCl, 0.15M NaCl, pH 7.6) for 5 minutes. The slides were then incubated for 20 minutes at room temperature (RT) with normal rabbit serum (DAKO, X092) diluted 1:5 in TBS to block non-specific binding. This was then removed and 25-30µl of optimally diluted primary antibody was placed on the cells. The slides and tissue-culture plates were placed on a tray containing moistened tissue paper and incubated at 37^0C for 2 hours. The primary antibodies used in the study are listed in Table 2.7.3. The slides were then rinsed in TBS/ 0.1% Tween (Sigma, P-1379), x3 in 15 min, and then incubated for 30 min with biotinylated rabbit anti-mouse immunoglobulins (DAKO, E354) diluted 1:300 in TBS. The slides were rinsed as before and incubated with strepABComplex/ Horse Radish Peroxidase (HRP) (DAKO, K377) for 30 min at RT, after they were rinsed x3 in TBS/ 0.1% Tween in 15 min. The cells were then incubated with a DAB solution (DAKO, S3000) for 10-15 min. Excess DAB solution was then rinsed off with UHP water and the slides were counter-stained with a 3% methyl green (Sigma, M-5015). Slides were then mounted using a commercial mounting solution (DAKO, S3023).

Antibody	Dilution	Supplier	Catalogue No.
323/A3 (anti-Ep-CAM) *	1 : 150	NeoMarkers	MS-181-P1
Ep-CAM Ab1 (VU-1D9) *	1 : 150	NeoMarkers	MS-144-P1
Cytokeratin-8	1 : 200	Sigma	C 5301
Cytokeratin-18	1 : 800	Sigma	C 8541
Cytokeratin-19	1 : 50	Sigma	C 6930
Pan-Cytokeratin	1 : 100	Sigma	C 2562
B_1-Integrin	1 : 100	Serotech	MCA 1188

* These anti-bodies were used together to increase sensitivity.

Table 2.7.3 Primary antibodies used in immunocytochemical studies.

2.7.4 DIFFERENTIATION STUDIES. WESTERN BLOT ANALYSIS.

Proteins for western blot analysis were separated by SDS-polyacylamide gel electrophoresis (SDS-PAGE).

2.7.4.1 Sample preparation

Cells were inoculated into $75cm^2$ flasks at a density of $1x10^5$ cells per flask and allowed to attach and form colonies. Medium, at concentrations from 0-10μM, was then added to the cells after 48 hours. The medium was then removed and replaced with medium with the required concentrations of halogenated thymidine analogue, or with HSM. Flasks were wrapped in aluminium foil because of the light-sensitive nature of some of the compounds being used e.g. oestrogen. The flasks were incubated for 7 and 14 days. Medium was replaced every 2-3 days over the course of the assay. All waste medium was retained for disposal by incineration. The cells were then harvested by trypsinisation, washed three times in cold, sterile PBS A, pelleted and stored at -80^0C until required.

Cells were then lysed in buffer containing 62.5mM Tris-HCl pH 6.8, 12.5% glycerol, 2% Nonidet P40 (Sigma, N6507), 2.5mM phenylmethylsulphonyl fluoride (PMSF) (Sigma, P7626), 1.25mM EDTA, 12.5μg/ml leupeptin (Sigma, L2884), 116μg/ml aprotinin (Sigma, A1153) for 30 min on ice. The extracts were used immediately for western blot analysis.

2.7.4.2 Gel electrophoresis

Resolving and stacking gels were prepared as outlined in Table 2.7.4 and poured into clean 10cm x 8cm gel cassettes which consisted of 1 glass and 1 aluminium plate, separated by 0.75cm plastic spacers. The resolving gel was poured first and allowed to set. The stacking gel was then poured and a comb was placed into the stacking gel in order to create wells for sample loading. Once set, the gels could be used immediately or wrapped in aluminium foil and stored at 4^0C for 24 hours.

Before samples were loaded onto the stacking gels, equal cell numbers (usually $2x10^4$ cells per lane) were further lysed in 2x loading buffer (2.5ml 1.25M-Tris/HCl, 1.0g SDS, 5.8ml glycerol and 0.1% bromophenol blue (Sigma, B8026) made up to 25ml with distilled water). The samples were then loaded including 6µl of molecular weight colour protein markers (Sigma, C-3437). The gels were run at 250V, 45mA for approximately 1.5 hours. When the bromophenol blue dye front was seen to have reached the end of the gels, electrophoresis was stopped.

Components	Resolving gel (7.5%)	Resolving gel (12%)	Stacking gel
Acrylamide stock*	3.8ml	5.25ml	0.8ml
Ultrapure water	8.0ml	6.45ml	3.6ml
1.875M-Tris/HCl, pH 8.8	3.0ml	3.0ml	-
1.25M-Tris/HCl, pH 6.8	-	-	0.5ml
10% SDS (Sigma, L-4509)	150µl	150µl	50µl
10% Ammonium persulphate (Sigma, A-1433)	60µl	60µl	17µl
TEMED (Sigma, T-8133)	10µl	10µl	6µl

* Acrylamide stock = 29.1g acrylamide (Sigma, A-8887) and 0.9g NN'-methylene bis-acrylamide (Sigma, N-7256) made up to 100ml with distilled water

Table 2.7.4. Preparation of electrophoresis gels

2.7.4.3 Western blotting

Following electrophoresis, the acrylamide gels were equilibrated in transfer buffer (25mM Tris, 192mM glycine (Sigma, G-7126) pH 8.3-8.5 without adjusting) for 20 min. Protein in gels were transferred onto Hybond ECL nitrocellulose membranes

(Amersham, RPN 2020D) by semi-dry electroblotting. Five sheets of Whatman 3mm filter paper (Whatman, 1001824) were soaked in transfer buffer and placed on the cathode plate of a semi-dry blotting apparatus. Excess air was removed from between the filters by moving a glass pipette over the filter paper. Nitrocellulose, cut to the same size of the gel, was soaked in transfer buffer and placed over the filter paper, making sure there were no air bubbles. The acrylamide gel was placed over the nitrocellulose and five more sheets of pre-soaked filter paper were placed on top of the gel. Excess air was again removed by rolling the pipette over the filter paper. The proteins were transferred from the gel to the nitrocellulose at a current of 34mA at 15V for 23 min.

All incubation steps from now on, including the blocking step, were carried out on a revolving apparatus to ensure even exposure of the nitrocellulose blot to all reagents.

The nitrocellulose membranes were blocked for 2 hours at room temperature with fresh filtered 5% non-fat dried milk (Cadburys; Marvel skimmed milk) in TBS/ 0.1% Tween. pH 7.5.

After blocking, the membranes were rinsed with PBS A and incubated with primary antibody (table 7.2.5) overnight at 4^0C. The primary antibody was removed and the membranes rinsed 3 times with TBS/ 0.1% Tween. The membranes were then washed for 15 min, and then twice for 5 mins in TBS/ Tween. Bound antibody was detected using enhanced chemiluminescence (ECL).

Antibody	Dilution	Supplier	Catalogue No.
323/A3 (anti-Ep-CAM) *	1 : 300	NeoMarkers	MS-181-P1
Ep-CAM Ab1 (VU-1D9) *	1 : 300	NeoMarkers	MS-144-P1
Cytokeratin-8	1 : 400	Sigma	C-5301
Cytokeratin-18	1 : 1000	Sigma	C8541
Cytokeratin-19	1 : 300	Sigma	C-6930
B_1 Integrin	1 : 10,000	Chemicon	AB1937

Table 2.4.5 Primary antibodies used in western blot analysis.

2.7.4.5 Enhanced chemiluminescence detection

Protein bands were developed using the Enhanced Chemiluminescence Kit (ECL) (Amersham, RPN2109) according to the manufacturer's instructions.

Secondary antibody (1/1,000 dilution of anti-mouse IgG peroxidase conjugate (Sigma, A-6782) in TBS) was added for 1 hour. The secondary antibody was removed and the membranes were washed as before. A sheet of parafilm was flattened over a smooth surface, *e.g.* a glass plate, making sure all air bubbles were removed. The membrane was then placed on the parafilm, and excess fluid removed. 1.5ml of ECL detection reagent 1 and 1.5ml of reagent 2 were mixed and covered over the membrane. Charges on the parafilm ensured the fluid stayed on the membrane. The reagent was removed after one minute and the membrane wrapped in cling film. The membrane was exposed to autoradiographic film (Kodak; X-OMAT S, 500 9907) in an autoradiographic cassette for various times, depending on the

signal. The autoradiographic film was then developed.

The exposed film was developed for 5min in developer (Kodak, LX24) diluted 1:6.5 in water. The film was briefly immersed in water and transferred to a Fixer solution (Kodak, FX-40) diluted 1:5 in water, for 5min. The film was transferred to water for 5 min and then air-dried.

2.8 RNA EXTRACTION

For all procedures using RNA, most glassware, solutions and plastics were treated with 0.1% diethyl pyrocarbonate (DEPC) before use, which is a strong inhibitor of RNases.

RNA was extracted from the cells as follows:
Cells were seeded into 175cm^2 flasks (Nunc, I-56502A) at a density of approximately 2x10^6 per flask and allowed to attach and form colonies for 48-72 hours at 37°C. The flasks were then treated with BrdU for time periods of 7 and14 days. The cells were trypsinised and the pellet was washed once with PBS A. The cells were pelletted and lysed using 1ml of TRI REAGENT^{TM} (Sigma, T-9424). The samples were allowed to stand for 5 min at RT to allow complete dissociation of nucleoprotein complexes. 0.2ml of chlorofom was then added per ml of TRI REAGENT^{TM} used and the sample was shaken vigorously for 15 sec and allowed to stand for 15 min at RT. The sample was then centrifuged at 13000rpm for 15 min at 4°C. This step separated the mixture into 3 phases; the RNA was contained in the colourless upper aqueous layer. This layer was then transferred to a new Eppendorf and 0.5ml of isopropanol was added. The sample was mixed and allowed to stand at RT for 10 min before being centrifuged at 13000rpm for 10 min at 4 C. The RNA formed a precipitate at the bottom of the tube. The supernatant was removed and the pellet was washed with 1ml of 75% ethanol and centrifuged at 4°C for 5 min at 13000rpm. The supernatant was removed and the pellet was briefly allowed to air-dry. 20-30µl of DEP-C water was then added to the RNA to resuspend the pellet.

RNA concentration was calculated by determining its OD at 260nm and 280nm and using the following formula:-

OD_{260nm} x Dilution factor x 40 = µg/ml RNA

The purity of the RNA extraction was calculated by determining its OD at 260nm and 280nm. An A_{260nm} : A_{280nm} ratio of 2 is indicative of pure RNA. Only those samples with ratios between 1.7 and 2.1 were used.

2.9 REVERSE TRANSCRIPTASE REACTION

Reverse transcriptase (RT) reactions were carried out in laminar flow cabinets using micropipettes, which were specifically allocated to this work.

cDNA was formed using the following procedure:-

1µl oligo $(dT)^{12-18}$ primers (1µg/µl) (Promega; C1101)

1µl total RNA (1µg/µl) (see 2.20)

3µl water

Were mixed in a 0.5ml Eppendorf (Eppendorf, 0030 121.023), heated to 70°_C for 10 min and then chilled on ice. To this, the following were added:-

4µl of a 5x buffer (250mM-Tris/HCl pH 8.3, 375mM-KCl and 15mM-$MgCl_2$)

2µl DTT (100mM) (Gibco; 510-8025 SA)

1µl RNasin (40U/µl) (Promega; N2511)

1µl dNTPs (10mM of each dNTP)

6µl water

1µl Moloney murine leukaemia virus-reverse transcriptase (MMLV-RT) (40,000U/µl) (Gibco; 510-8025 SA).

The solutions were mixed and the RT reaction was carried out by incubating the Eppendorfs at 37°C for 1 hour. The MMLV-RT enzyme was then inactivated by heating to 95°C for 2 min. The cDNA was stored at -20°C until required for use in PCR reactions as outlined in Section 2.10.

2.10 POLYMERASE CHAIN REACTION

A standardised polymerase chain reaction (PCR) procedure was followed in this study. The Eppendorf tubes used (Eppendorf, 0030 121 023) and the sterile water were DEPC-treated. All reagents had been aliquoted and were stored at –20°C and all reactions were carried out in a laminar flow cabinet.

Each PCR tube contained the following:-

24.5µl water

5µl 10x buffer* (100mM-Tris/HCl, pH 9.0, 50mM-KCl, 1% Triton X-100)

3µl 25mM-$MgCl_2$*

8µl dNTPs (1.25mM each of dATP, dCTP, dGTP and dTTP) (Promega; U1240)

1µl each of first and second strand target primers (250ng/ml) (keratin 19 - Table 2.22.1)

1µl each of first and second strand endogenous control primer (250ng/ml) (β-actin)

0.5µl of 5U/µl *Taq* DNA polymerase enzyme*

5µl cDNA

*(Promega; N1862)

A drop of autoclaved mineral oil was placed in each reaction tube to prevent evaporation and the DNA was amplified by PCR as follows:

95°C for 1.5 min - to denature double-stranded DNA

30 cycles: 95°C for 1.5 min. - denature

55°C for 1 min - anneal

72°C for 3 min. - extend

72°C for 7 min. - extend

The reaction tubes were then stored at 4°C until analysed by gel electrophoresis as described in Section 2.13.

2.11 GUIDELINES USED IN THE SELECTION OF PRIMERS.

To successfully amplify cDNA by PCR, specific primers to the gene of interest must be chosen to prime the cDNA for PCR amplification, a number of guidelines should be followed, when feasible, to design the primer pair to give successful amplification of a PCR product of suitable size.

- **Specificity to the gene of interest:** The fundamental requirement for a primer is that it should hybridise efficiently to the sequence/genes of interest with negligible hybridisation to other sequences/genes present in the sample. To find the most homologous sequences to be used as primers, DNA bases (such as the one held by EMBL) must be searched.
- **Target length:** The distance between the primers for which optimum amplification can be achieved is generally considered to between 180 and 500 bp. However, much longer targets may be amplified efficiently.
- **Primer length and base composition:** The length of a primer contributes to its specificity. It is generally considered ideal to choose oligonucleotide primers between 18 and 30 bases in length; however, shorter and longer primers will work. Long stretches of purines or pyrimidines must also be avoided. If possible primers should have a balanced G/C and A/T concentration and the distribution of bases should be as random as possible. These precautions are required to avoid areas of secondary structure formation or complimentarily between primers.
- **Annealing temperature:** The annealing temperature of primers is determined by their length and base composition. Increasing this temperature enhances discrimination against incorrectly annealing primers and so reduces the extension of incorrect nucleotides at the 3' end of the primer. It is considered advisable to choose primers whose melting temperature (T_m) (i.e. the temperature at which half of the duplex is dissociated are between 55°C and 75°C. The T_m for a primer can be estimated from the following equation:

T_m=2°C (no. of A+T residues) 4°C (no. of G+C residues)

Gene	Primer Sequence/cDNA position	Product size/ Reference
Cytokeratin-8	AAC AAC CTT AGG CGG CAG CT (cDNA position 449-468)	244 bp Burchill *et al.*, 1995
	GCC TGA GGA AGT TGA TCT CG (cDNA position 673-692)	
Cytokeratin-18	CAA GAT CAT CGA AGG ACC TG (cDNA position 436-454)	444bp designed from corresponding rat primers in Fridmacher *et al.*, 1995
	CTC TCC TCA ATC TGC TGA GA (cDNA position 860-879)	
Cytokeratin-19	GCG GGA CAA GAT TCT TGG TG	Burchill *et al.* (1995)
	CTT CAG GCC TTC GAT CTG CAT	
Ep-CAM (GA733-2)	CTG TCA TTT GCT CAA AGC TG (cDNA position 167-186)	368bp Designed at NCTCC
	TGG ATC CAG TTG ATA ACG (cDNA position 517-534)	
GA733-1	CAC ACC GAC GTC TGG TTG CA (cDNA position 355-374)	164bp Designed at NCTCC
	TCC AAG TGT CTG CTG CTC A (cDNA position 211-229)	

Table 2.8.1 Primer sequences used in RT-PCR reactions.

2.12 USING cDNA SEQUENCE DATABASES TO CHECK UNIQUENESS OF PRIMERS (BLAST).

The most critical element in choice of primers for PCR is obviously that the primers were unique *i.e.* that they did not cross-react with sequences likely to be present in the mRNA of the cells/tissues being studied. This involved choosing primers using the criteria described in Section 2.11, and checking *via* a DNA database (*e.g.* EMBL, GenBank) what similar sequences existed. This approach is essential if no references are available on RT-PCR of the mRNA in question. Even where references do exist, there is need for checking because:

I. The cDNA data-base is expanding rapidly and new cross-reactions may have been discovered since the choice of primers was published;

II. Occasionally, published primers have not been well chosen and cross-reactions have been overlooked; also, occasionally, probably due to typographical errors, incorrect primer sequences are published.

III. Because of the excellent search facilities available, checking sequence uniqueness of primer sequences is a straightforward procedure.

DNA databases were accessed *via* the Internet by linking to large mainframe computers (*e.g.* VAX) and connecting to a UNIX system run by the Irish National Centre for BioInformatics, Trinity College, Dublin.

- A mRNA or cDNA sequence of interest can be extracted from the database once its name(s) is known. (If the accession number or sequence deposition number are known these can be used to in a similar way). The following is an example of how to access a mRNA sequence through this system.

LOCATION: **http://www3.ncbi.nlm.nih.gov/Entrez/nucleotide.htm**

Then enter name of the sequence required e.g. **GA733-1 [Enter]**

The response to this is a list of sequences submitted which have GA733-1 as part of their name - including full and partial cDNA sequences from various species of origin. The sequence required for these studies was identified in this list as "human mRNA for pancreatic carcinoma marker GA733-1"; accession number: X13425.

To reveal the complete mRNA sequence, the option **GenBank Report** was selected.

- BLAST (Basic Local Alignment Search Tool), a set of similarity search programs designed to explore all of the available sequence data-bases was used for homology searching of primers being considered as potentially suitable. The following is an example of how such a search was done to identify suitable primers.

LOCATION: **http://acer.gen.tcd.ie**

Select option: **BioInf Servers**

Select option: **US (NCBI) Servers: Blast server at NCBI**
(An option to check the European (EBI) server, Fasta, is also available at this stage. Either the US or European servers may be used for access as they both link to the same databases. However, before finally selecting primers it is wise to check *via* both servers to ensure that all relevant information is accessed).

Select option: **Advance BLAST search**

A window then becomes available to enter the primer sequence to be checked. Alignment and description of the (first 0, 10, 50, 100 or 500) sequences with strongest homology may be requested.

Select option: **Search**

Depending on how busy the system is the results may be returned immediately or after some time. The option is available to enter an e-mail address to which the results will be posted.

2.13 ELECTROPHORESIS OF PCR PRODUCTS

A 3% agarose gel (NuSieve, GTG) was prepared in TBE buffer (5.4g Tris, 2.75g boric acid, 2ml 0.5M-EDTA pH 8.0 in 500ml water) and melted in a microwave oven. After allowing to cool, 0.003% of a 10mg/ml ethidium bromide solution was added to the gel, which was then poured into an electrophoresis apparatus (BioRad). Combs were placed in the gel to form wells and the gel was allowed to set.

10µl loading buffer (50% glycerol, 1mg/ml xylene cyanol, 1mg/ml bromophenol blue, 1mM EDTA) was added to each 50µl PCR sample and 20µl was run on the gel at 80-90mV for approximately 2 hours. When the dye front was seen to have migrated the required distance, the gel was removed from the apparatus and examined on a transilluminator and photographed.

2.14 ISOLATION OF RAT TYPE II PNEUMOCYTES.

2.14.1 ANIMALS AND ANAESTHESIA.

2.14.1.1 Animals.

Male Sprague-Dawley rats of 180 - 200 g were used in the experiments. The animals were allowed water and food *ad librium*.

2.14.1.2 Anaesthesia.

The animals were lightly anaesthetised with halothane prior to receiving a lethal intraperitioneal injection of pentobarbital (60 mg/kg = 1ml/kg). The animal was laid on its back and the areas to be incised dampened with IMS. The ventral surface skin was removed and the abdominal vessels exposed. The major dorsal blood vessels were cut through with a large straight scissors.

2.14.2 DISSECTION AND PERFUSION.

The trachea was cannulated and the chest opened without puncturing the lungs. A portion of the thymus was removed to expose the heart. An incision was made into the pericardium and a cannula fed into the pulmonary artery. The cannula was connected by gravity feed to a reservoir of 0.15 mol/l NaCl, which upon flowing increased the size of the right atrium. The atrium was cut to allow fluid to drain free. The lungs were artificially ventilated with 8 to 10mls of air by means of a syringe. This was repeated 5 times, after which the lungs were free of blood and white in appearance.

2.14.3 LAVAGE AND DIGESTION.

2.14.3.1 Lavage.

The lungs were dissected free and lavaged exactly 5 times with 6-8 ml of 0.15 mol/l NaCl solution. The lavage fluid was removed from the lungs by gravity, this fluid contained lung macrophage population.

2.14.3.2 Digestion.

The lung were digested using crystalline trypsin (Sigma T8003; 250 mg/ 100ml) dissolved in +Mg/Ca solution The trypsin was perfused into the lungs via the cannula, so that the lungs were continuously filled with trypsin solution (using about 60 ml per lung). The lungs were allowed to digest for 30 minutes. The lobes were dissected from the airways and cut into cubes of 1 - 2 mm3 with a scissors. These were added to 5 ml of FCS and the total volume was brought to 20 ml by the addition of DNase solution I (250 μg/ml in –Mg/Ca solution). The suspension was shaken for 5 minutes prior to filtering through a course sterile wire mesh (stainless steel tea strainer) and then through two sterile nylon filters (Falcon) of 100 μm and 30 μm respectively.

- **Mg/Ca Solutions**

Stock solutions:-

0.9%w/v	NaCl	
0.11M	CaCl	
0.15M	KCl	
0.10M	Phosphate buffer	
	0.10M	Na_2HPO_4
	0.10M	NaH_2PO_4
	pH to 7.4 by adding HaH2PO4 to Na2HPO4	
0.20M	HEPES	
0.15M	$MgSO_4$	

	+Mg/Ca Solution	-Mg/Ca Solution
0.9%w/v NaCl	250ml	250ml
0.11M CaCl	5ml	
0.15M KCl	10ml	10ml
Phosphate Buffer	7.5ml	7.5ml
HEPES	15ml	15ml
0.15 $MgSO_4$	2.5ml	
Glucose	3.15mg	315mg

Table2.13.1 Recipe for Mg/Ca solutions for isolation of type II pneumocytes from 1 rat.

2.14.4 PERCOLL GRADIENT CENTRIFUGATION AND DIFFERENTIAL CELL ATTACHMENT.

2.14.4.1 Percoll Gradient Centrifugation.

To purify the primary digest, which contains a mixture of cell types a density

centrifugation with percoll was performed. The cell suspension was layered upon two percoll layers: a heavy percoll solution [65 % percoll (1.089 g/ml)] and a light percoll solution [27 % percoll (10.39 g/ml)]. The discontinuous density centrifugation was carried out at a speed of 1,850 rpm for 20 minutes at 10 °C. Following centrifugation the fraction located at the interface between the heavy and light percoll layers, which contains an enriched fraction of type II pneumocytes was carefully removed.

2.14.4.2 Differential Cell Attachment.

The removed fraction was brought to a volume of 40 ml by the addition of DNase solution II. This cell suspension was spun at 1,000 rpm for 10 minutes. The cell pellet was re-suspended in growth medium, ATCC supplemented with 5% v/v FCS, 2mM L-glutamine, 2% Penicillin-Streptomycin (Sigma Cat. No. P4458), 0.5% v/v Fungizone and plated on tissue culture plastic for two hours at 37 °C. The non-adherent cells were gently removed and centrifuged. The resulting pellet was re-suspended and a cell yield obtained. This cell suspension was then plated at the required density for the experiment.

2.15 PRIMARY CULTURE OF HUMAN LUNG TUMOURS.

2.15.1 COLLECTION OF TUMOUR TISSUE SAMPLES AND TRANSPORTATION MEDIUM.

Primary tissue was collected in theatre after surgical resection at St. Vincents Hospital, Elm Park, Stillorgan, Dublin 4. This material was kindly provided by thorasic surgeon Mr. Vincent Lynch only after the requirements of the hospital's histopathology service for such tissue has been met. Tissue samples were placed in a transportation medium immediately and taken at 4 °C to the NCTCC at DCU for processing on the same day.

- **Transportation Medium:**
  This consisted of Leibovitz L-15 medium as basal medium. As this media is bicarbonate free there is minimal pH fluctuation during transit. This was supplemented with 1% w/v of PVP-360, 2 mM L-glutamine, 1% v/v Penicillin-Streptomycin (Sigma Cat. No. P4458) and 0.5%v/v fungizone.

2.15.2 DISSECTION OF TUMOUR TISSUE.

All procedures from this point on were performed in a class 2 laminar flow cabinet. Surgical quality gloves were worn and due care and diligence was taken in the disposal of any waste.

Samples were rinsed in PBS-A 3 times to remove extraneous blood prior to dissection. Fatty deposits, necrotic tissue and blood vessels were removed where possible using sterile surgical scalpels and scissors. These manipulations were performed on sterile petri dishes.

2.15.3 ENZYMATIC DIGESTION.

The remain tissue was cut in to small chunks 3 - 4 mm^3. These tissue fragments were placed in a universal and washed once with PBS-A by inversion. The tissue was then incubated with proteolytic enzyme mixture A or B, while stirring with a sterile magnetic stirrer at 37 °C.

After 30 minutes the supernatant was removed from the digest and the cells recovered by centrifugation at 1000 rpm for 10 minutes. The pellet was resuspened in prewarmed growth media.

The remaining tissue fragments from the digest were reincubated with fresh proteolytic enzyme for a further 30 minutes. The supernatant from the second digest was treated as above.

The cell suspensions from both digests were pooled and centrifuged at 1000 rpm for 10 minutes. The resulting cell pellet was resuspended in prewarmed growth media and plated at high density, i.e. 10^6 cell per $25cm^2$ flask.

- **Proteolytic enzyme mixture A:**
  Consisted of trypsin 2ml of 10x Gibco BRL trypsin in 18 ml of MEM supplemented with 10U/ml of DNase I.
- **Proteolytic enzyme mixture B:**
  Consisted of 0.4mg/ml Collagenase A, 0.6mg/ml Dispase, 0.6mg/ml Pronase E in MEM supplemented with 10U/ml of DNase I.

2.15.4 EXPLANT TECHNIQUE.

Lung tumour tissue was cut into small cubes (2-4mm^3) these were aseptically transferred to the growth surface of $25cm^3$ flasks. These flasks had been pre-wetted with 2.5mls of growth medium (which contained serum) and the excess media was removed. This was performed 10-15 minutes prior to the placing of explant tissue in

the flask. Approximately 15-20 fragments of tissue were carefully placed in the flask with a scalpel blade or sterile tweezers. The tissue was allowed to adhere in this position for approximately 30 minutes with the lids slightly loosened. Taking care not to dislodge the explants, 2.5mls of growth medium was added. The flasks were then incubated at 37°C.

2.15.5 DIFFERENTIAL TRYPSINISATION FOR THE REMOVAL OF FIBROBLASTS.

The procedure utilises the phenomenon that fibroblast cells detach first in a mixed culture treated with trypsin. Thus the presence of contaminating fibroblasts in a culture can be reduced or eliminated by the trypsinisation the mixed culture.
All operations were performed aseptically in a laminar flow. The culture media was removed from the flask and pre-warmed trypsin EDTA solution pipetted into the flask and the lid replaced. The flask was placed on an inverted microscope stage and the progress of the trypsinisation monitored continuously under 40x and 100x magnification.

As soon as the fibroblasts had detached but before the cells of interest, the flask was returned to the laminar flow. Avoiding unnecessary agitation of the flask, growth media was gently added to terminate the trypsinisation procedure. This solution which now contains the fibroblast cells, was gently removed by aspiration and fresh growth medium added.

2.16 TIME-LAPSE VIDEO MICROSCOPY.

Time-lapse video microscopy was carried out on a Nikon diahot inverted microscope (Micron Optical, Bray, Ireland) equipped with a phase contrast optics, linked to a Mitsubishi CCD-100 colour video camera. Images were recorded in S-VHS onto a Mitsubishi HS-S5600 video recorder with time-lapse capabilities. All time-lapse video-equipment was obtained from Laboratory Instruments (Ashborne, Ireland). Recording speed was set at 3.22sec/field (480 hour mode), which at normal playback speed resulted in an acceleration factor of 160.

The temperature of the culture vessel was controlled by a Linkam C0102 warm stage controller. This controller was adjusted to keep the culture medium inside the vessel at 37 C, as measured using a TB3301 probe.

3.0 RESULTS.

3.1 INVESTIGATION OF THE EFFECT OF BrdU EXPOSURE ON Ep-CAM EXPRESSION IN THE HUMAN LUNG CARCINOMA CELL LINES DLKP AND A549.

Previous studies in this laboratory have shown that 10μM 5,2'-Bromodeoxyuridine (BrdU) induces cytokeratin (CK) 8, and 18 expression in DLKP and enhances their expression in A549 (McBride *et al*, '99). BrdU also induces CK-19 expression in DLKP and enhanced expression in A549 (Meleady *et al*, manuscript in preparation). Induced α_1 and α_2 integrin and enhanced β_1 expression has been observed in both DLKP and A549 (Meleady and Clynes, manuscript submitted).

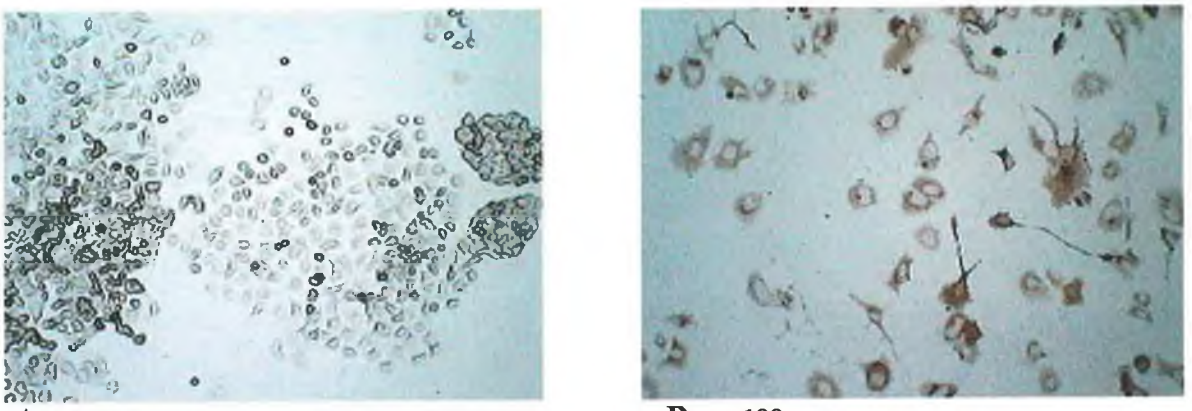
Ep-CAM is a transmembrane protein that is expressed on the baso-lateral domains of epithelial cell membranes. It is a homophilic cell-cell adhesion protein and is believed to play role in cell sorting during development. Preliminary results (Dr. Paula Meleady, PhD thesis, DCU 1997) suggested that Ep-CAM was induced following treatment with 10μM BrdU in both DLKP and A549. Studies were thus conducted to further investigate this apparent induction.

3.1.1 IMMUNOCYTOCHEMICAL ANALYSIS OF Ep-CAM EXPRESSION.

Immunocytochemistry was used to qualitatively investigate the changes in Ep-CAM in DLKP and A549 following treatment for 7 days with 10μM BrdU.

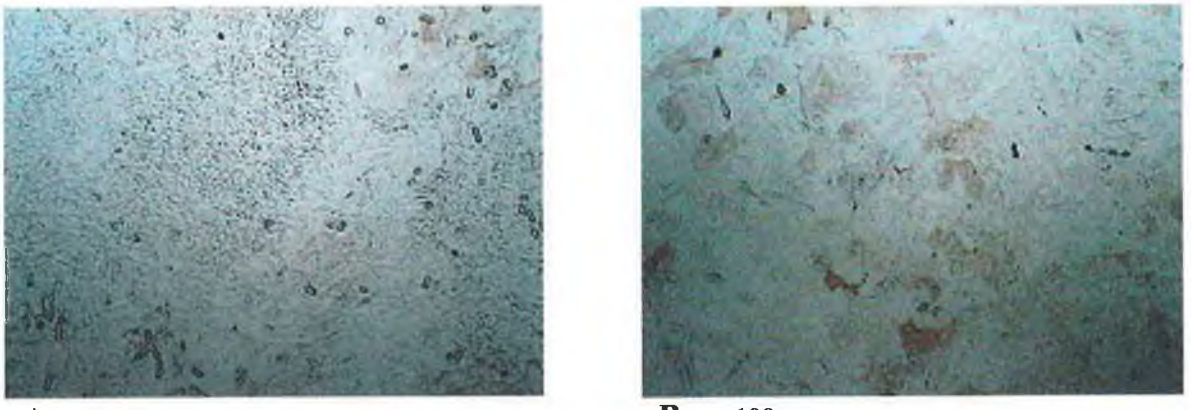
Untreated DLKP were found to be negative for Ep-CAM. Following treatment for 7 days with 10μM BrdU a faint staining was observed on the cell membrane in about 80% of the cell population with some cells showing strong staining (figure 3.1.1)

A549 cells that are untreated exhibit a slight positive staining for Ep-CAM. Following BrdU treatment this staining is strongly enhanced in approximately 70% of the cell population (figure 3.1.2).



A. x 100 B. x100

Figure 3.1.1 Ep-CAM expression on untreated DLKP (A) and BrdU treated DLKP (B)



A. x100 B. x100

Figure 3.1.2 Ep-CAM expression on untreated A549 (A) and BrdU treated A549 (B)

3.1.2 WESTERN BLOT ANALYSIS OF Ep-CAM EXPRESSION.

In order to obtain a quantitative assessment for BrdU induced expression of Ep-CAM in the lung cell lines DLKP and A549 western blot analysis was performed. Cells were treated for up to 21 days and time points taken at day 7, day 14 and day 21. The breast adenocarcinoma cell line MCF-7, which is known to express high levels of Ep-CAM, was used as a positive control.

In untreated DLKP cells no clearly visible band was detected in untreated cells. A faint band with the equivalent molecular weight for Ep-CAM (40-43 KDa.) was detected in cells treated for 7 days with BrdU. The intensity of this band increased in cells treated for 14 days with BrdU. In cells treated for 21 days with BrdU there was no apparent change in the intensity of the detected band compared to the band obtained for 14 days BrdU (figure 3.1.3).

In A549 a faint band (40-43 KDa.) equating to the molecular weight of Ep-CAM was detected in untreated cells. The intensity of this band increased slightly in cells treated over 14 days with 10μM BrdU. There was no apparent change in the intensity of the band obtained in cells treated for 21 days with BrdU compared to the band detected after 14 days treatment (figure 3.1.4).

The western blots for both cell lines showed unidentified lower molecular weight protein bands. Balzar *et al.* (1998) obtained a similar pattern of lower molecular weight products in their experiments, which they regarded as breakdown products of Ep-CAM.

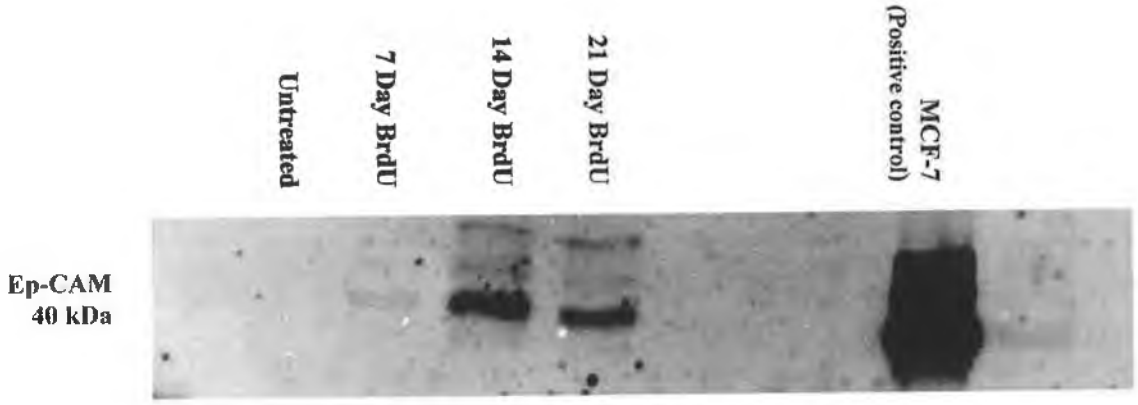


Figure 3.1.3 **Western blot analysis of Ep-CAM expression in DLKP treated with BrdU.**

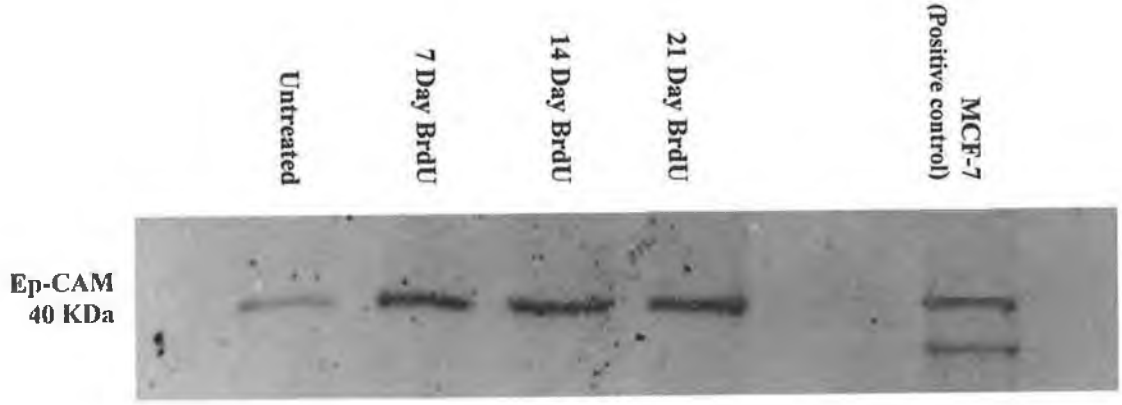


Figure 3.1.4 **Western blot analysis of Ep-CAM expression in A549 treated with BrdU.**

3.1.3 RT-PCR ANALYSIS OF Ep-CAM mRNA EXPRESSION FOLLOWING BrdU TREATMENT.

RT-PCR analysis was conducted to investigate the effect of BrdU treatment on the Ep-CAM (GA733-2) mRNA transcript in DLKP and A549. Time points of 7 days, 14 days, and 21 days of BrdU treatment were selected. The breast adenocarcinoma cell line MCF-7 was used as a positive control.

Optimal primers for the gene sequence were designed and the specificity was checked using BLAST (see methods section 2.11) (Table 3.1.1.).

	Sequence	Position on cDNA
Forward Primer	5' CTG TCA TTT GCT CAA AGC TG 3'	167-186
Reverse Primer	5' TGG ATC CAG TTG ATA ACG 3'	517-534

Table 3.1.1. Sequence of primers for Ep-CAM and their cDNA nucleotide position.

The primers selected amplified a product of 368 base pairs as predicted

No change in the level of mRNA transcript was detected in DLKP that were treated with BrdU for up to 21 days (figure 3.1.5). Similarly in A549 the level of Ep-CAM mRNA transcript remained unchanged over 21 days of BrdU treatment.

Thus in both cell lines the mRNA level was unchanged by treatment with 10μM BrdU though both showed induced Ep-CAM protein expression. This indicated that the regulation of Ep-CAM expression by BrdU is at the post-transcriptional level (possibly translational) level.

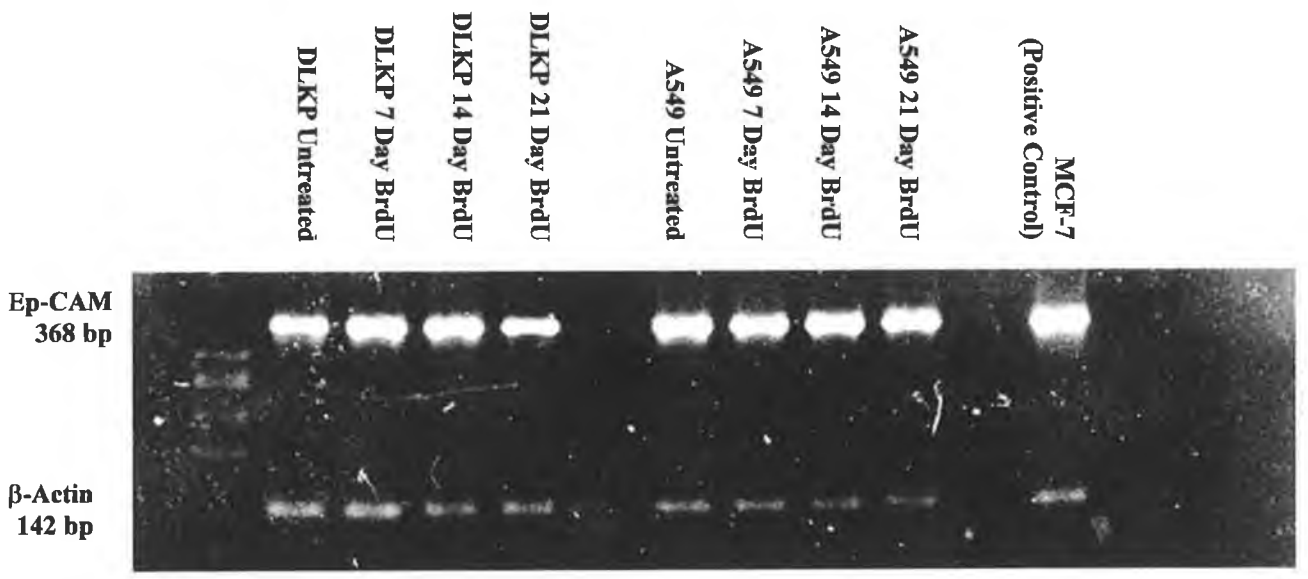


Figure 3.1.5 RT-PCR analysis of Ep-CAM expression in DLKP and A549 untreated and post- BrdU treatment.

3.1.4 INVESTIGATION OF THE mRNA LEVELS OF GA733-1 IN BrdU TREATED DLKP AND A549 CELLS.

RT-PCR analysis was conducted on 10μM BrdU treated DLKP and A549 to investigate if there was any change in the level of mRNA transcript for GA733-1 following BrdU treatment. Time points of 7 days, 14 days, and 21 days of BrdU treatment were selected. The breast adenocarcinoma cell line MCF-7 was used as a positive control.

Optimal primers for the gene sequence were designed and the specificity was checked using BLAST (see methods section 2.11) (Table 3.1.2).

	Sequence	Nucleotides
Forward Primer	5' TCC AAG TGT CTG CTG CTC A 3'	211-229
Reverse Primer	5' CAC ACC GAC GTC TGG TTG CA 3'	355-374

Table 3.1.2 Sequence of primers for GA733-1 and their cDNA nucleotide position.

The primers selected amplified a product of 164 base pairs as predicted.

In untreated DLKP no band equivalent to the amplified GA733-1 sequence was detected. In DLKP cells that had been treated for 7 days with BrdU a band was detected for GA733-1 product. The intensity of this band increased after 14 days of BrdU treatment, and remained unchanged in intensity after 21 days of BrdU treatment (figure 3.1.6).

In untreated A549 no amplified RT-PCR product was detected. In A549 cells that had been treated for 7, 14 and 21 days with BrdU there was also no detectable RT-PCR product (figure 3.1.6). Thus the induction appears to be DLKP specific, which is

unusal since induction is observed in both DLKP and A549 for most of the markers studied in this laboratory.

Unfortunately we were unable to source any GA733-1 specific antibodies to examine changes in the levels of the relevant protein.

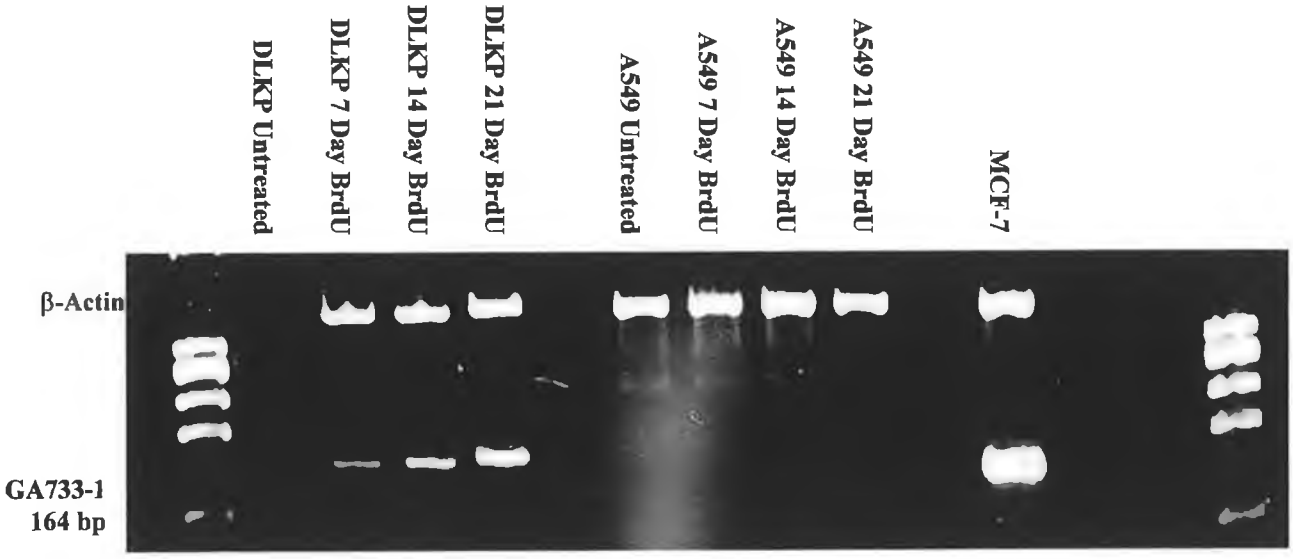


Figure 3.1.6 RT-PCR analysis of GA733-1 mRNA in DLKP and A549, pre- and post- BrdU treatment.

3.1.6 INVESTIGATION OF CHANGES IN FOCAL ADHESION PROTEINS IN BRDU TREATED DLKP AND A549.

It is believed that Ep-CAM may decrease E-cadherin mediated cell-cell binding by decreasing the level of the focal adhesion proteins α-catenin, β-catenin, and either sequestering or decreasing α-actinin.
Preliminary experiments were conducted using western blot analysis to investigate the changes in α-catenin and α-actinin following BrdU treatment and the induction of Ep-CAM. Time points were taken at 7 days, and 14 days of BrdU treatment.

In untreated DLKP a strong band was detected at the expected molecular weight for α-catenin. The intensity of this band dropped after 7 days of BrdU treatment and continued to drop over 14 days of BrdU treatment (figure 3.1.7).
Similarly, in A549 a strong band was detected in untreated cells for α-catenin, the intensity of which dropped over 14 days of BrdU treatment (figure 3.1.7)

A strong band for α-actinin was detected in untreated DLKP. The intensity of this band dropped after 7 days of BrdU treatment and continued to drop in intensity after 14 days of BrdU treatment. A similar pattern of decrease was observed in A549 (figure 3.1.8).

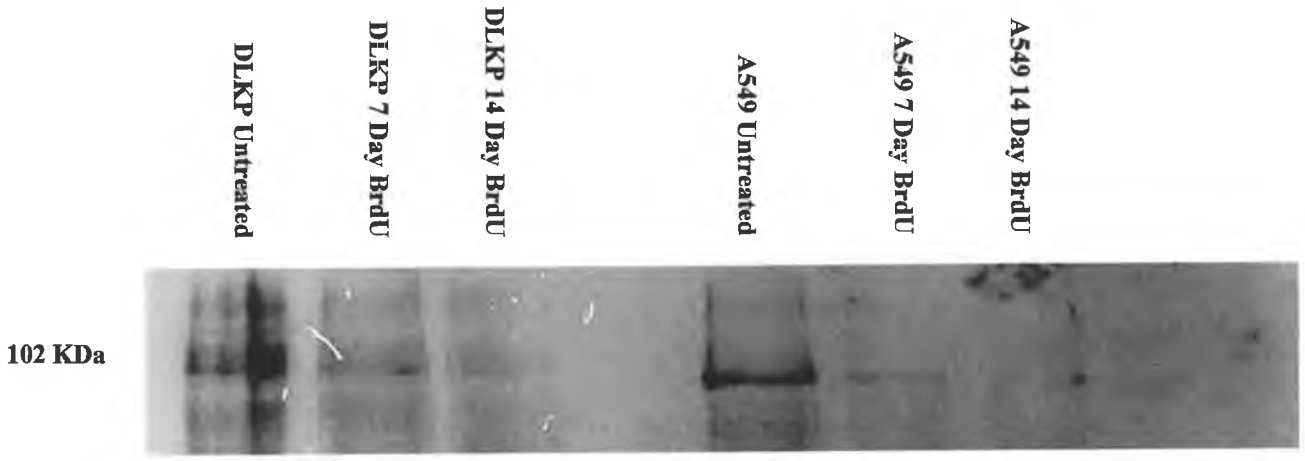


Figure 3.1.7 Western blot analysis of α-catenin in DLKP and A549 treated over 14 days with BrdU.

Figure 3.1.8 Western blot analysis of α-actinin in DLKP and A549 treated over 14 days with BrdU

3.2 HALOGENATED ANALOGUES OF THYMIDINE AND THEIR EFFECTS ON DIFFERENTIATION.

The halogenated thymidine analogue 5-bromo-2'-deoxyuridine has been shown to alter the differentiation status of various cell types. The effect of other halogenated thymidine analogues on differentiation is less well researched, with only a limited number of studies existing. It was decided to investigate the ability of other halogenated thymidine analogues to induce differentiation in the DLKP and A549 cell line models.

The following compounds were selected 5-chloro-2'-deoxyuridine (CdU), 5-fluro-5-deoxyuridine (5,5'-FdU), 5-bromouridine (5-BUr).

3.2.1 SELECTION OF CONCENTRATIONS FOR DIFFERENTIATION ASSAYS AND MORPHOLOGICAL EFFECTS OF THYMIDINE ANALOGUES ON DLKP AND A549.

3.2.1.1 Toxicity profiles of thymidine analogues on DLKP and A549.

Preliminary toxicity assays were performed to determine the toxic effect of CdU, 5,5'-FdU, and 5-BU. Figures 3.2.1 and 3.2.2 show the toxicity profiles obtained for DLKP and A549. Concentrations were chosen that would allow a 75% survival rate or greater (table 3.2.1). Both DLKP and A549 show a similar response profile to FdU and appear to have an IC_{50} around 3.5μM. DLKP and A549 exhibited different CdU profiles. DLKP appeared to have an apparent IC_{50} of 13.5μM, while A549 appeared to have an IC_{50} slightly greater then 30μM. However subsequent studies showed that CdU appeared to be cumulatively cytotoxic (data not shown), thus a lower concentration was chosen then the toxicity profiles suggested. Both DLKP and A549 5-BUr did not exhibit any toxicity to DLKP and A549 even at 70μM.

	μM
5,5'-FdU	**2**
CdU	**10**
5-BU	**70**

Table 3.2.1 Concentrations of halogenated analogues selected for differentiation assays.

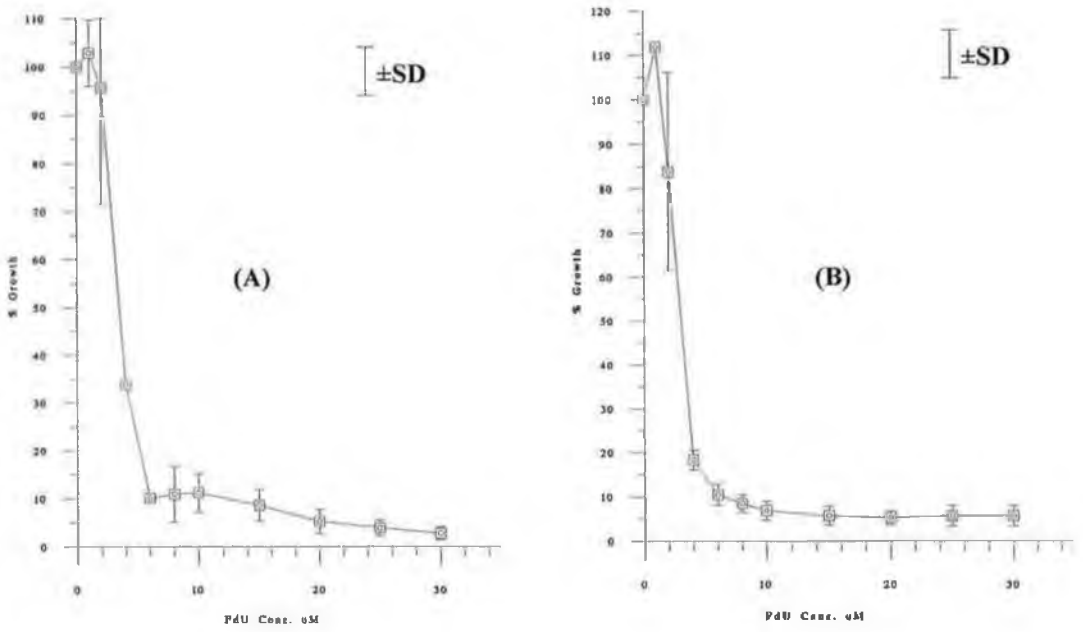


Figure 3.2.1 Toxicity profile of DLKP (A) and A549 (B) in 5.5'-FdU
(±SD = Standard Deviation)

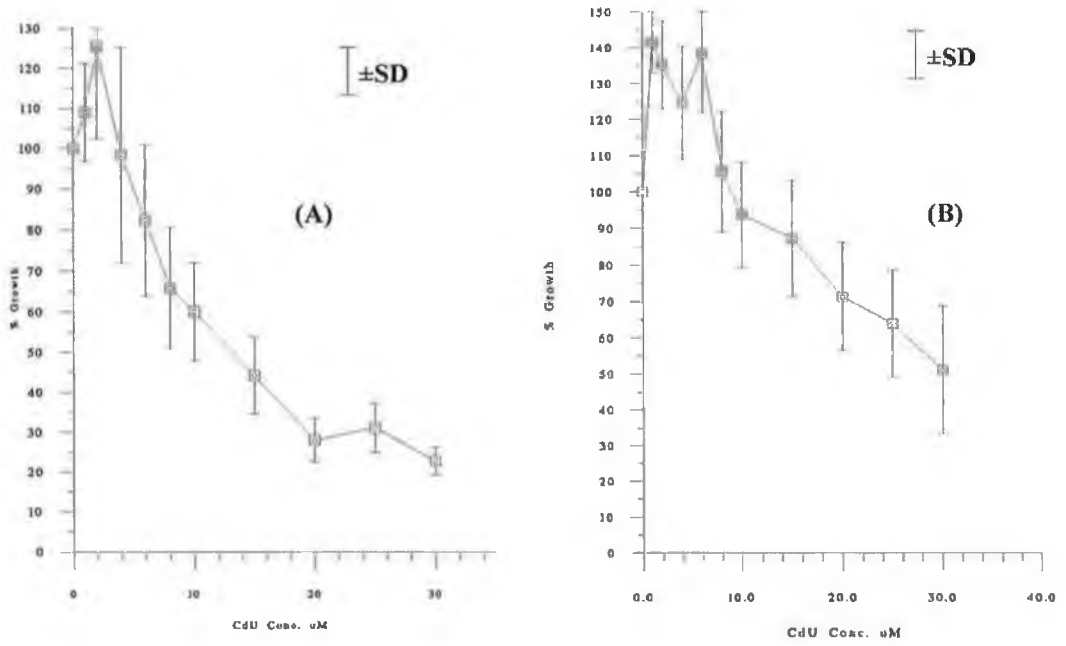


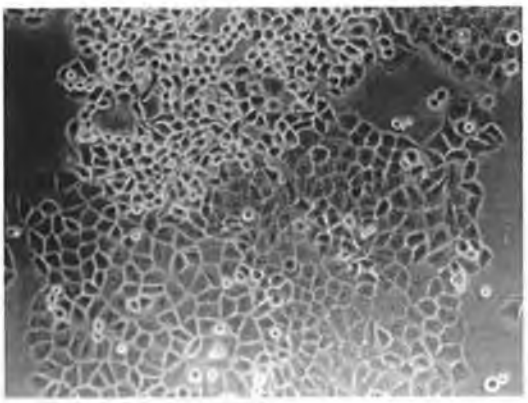
Figure 3.2.2 Toxicity profile of DLKP (A) and A549 (B) in CdU
(±SD = Standard Deviation)

3.2.1.2 Cellular morphology of A549 and DLKP exposed to thymidine analogues.

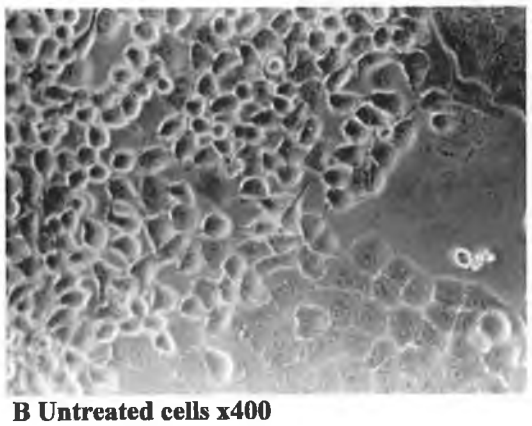
DLKP cells treated with 5,5'-FdU had a more spread morphology (figure 3.2.3 E and F) than the control DLKP cells (figure 3.2.3 A and B), which existed both as single cells and colonies. Though there was a change in cellular morphology following 5,5'-FdU treatment the degree of cell flattening and spreading was not as great as that for BrdU (figure 3.2.3 C and D).

DLKP cells treated with CdU were highly flattened and cells grew predominantly as single cells (figure 3.2.3 G and H). The change in cellular morphology of DLKP treated with CdU was reminiscent of the change in cell morphology that occurs in DLKP following BrdU treatment. However, as noted earlier as CdU treatment is extended beyond 7 days a cumulative cytotoxic effect occurs, unlike BrdU, which does not show any cumulative cytotoxicity. 5-BUr treatment did not appear to visibly alter the cellular morphology of DLKP (figure 3.2.3 I and J).

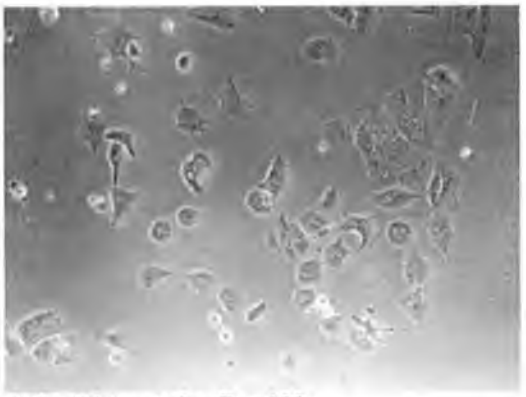
A549 cells treated with 5,5'-FdU exhibited a stretched morphology (figure 3.2.4 E and F) compared to untreated A549 (figure 3.2.4 A and B). The increased stretching and flattening in morphology is not as great as observed in BrdU treated cells (figure 3.2.4 C and D). CdU treatment of A549 induced extensive cellular flattening and cells grew predominantly as single cells (figure 3.2.4 G and H). The flattening in cellular morphology observed following CdU treatment was more extreme than in BrdU treated A549 cells (figure 3.2.4 C and D). 5-BUr treatment did not appear to visibly alter the cellular morphology of A549 (figure 3.2.4 I and J).



A. Untreated cells x100

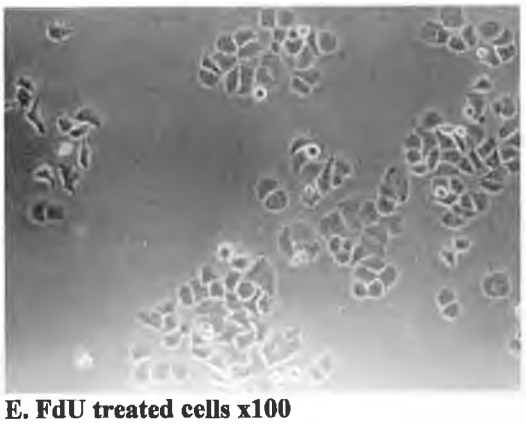


B Untreated cells x400

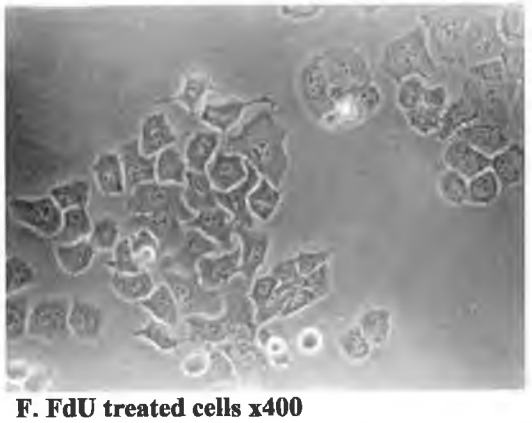


C. BrdU treated cells x100

D. BrdU treated cells x400

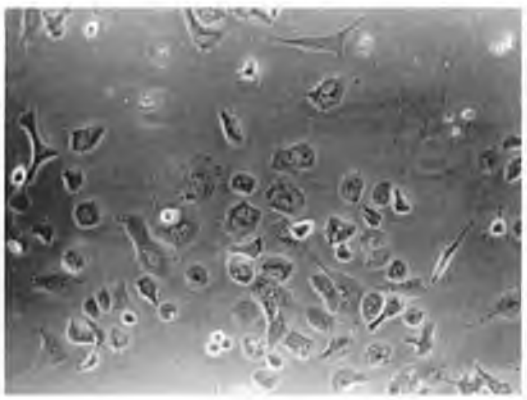


E. FdU treated cells x100



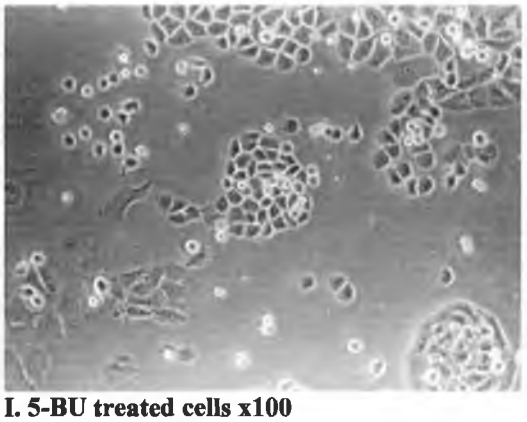
F. FdU treated cells x400

Figure 3.2.3 The effect of haolgenated thymidine analogues on the cellular morphology of DLKP.

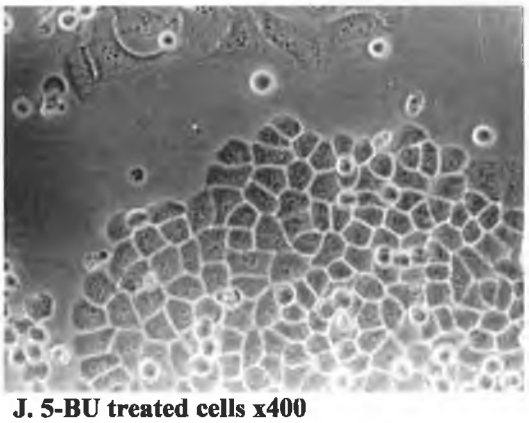


G. CdU treated cells x100

H. CdU treated cells x400

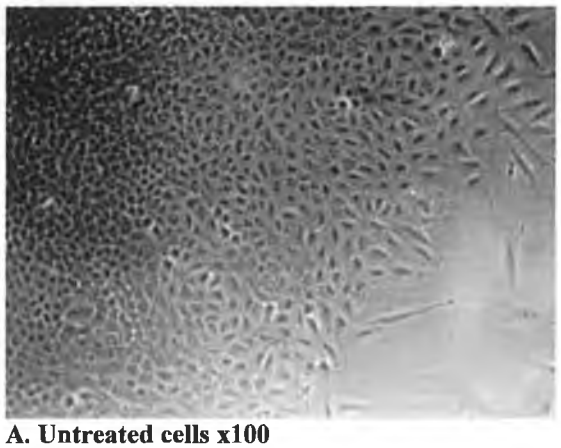


I. 5-BU treated cells x100

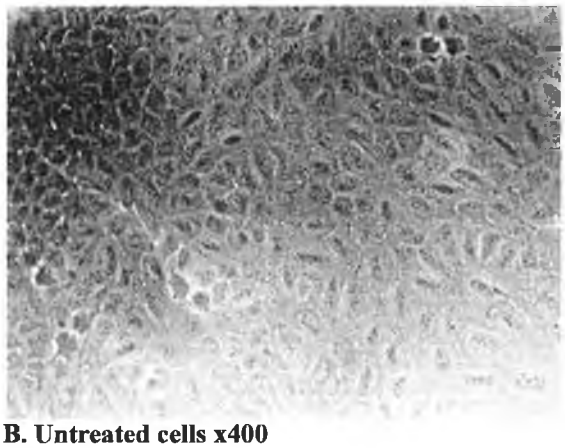


J. 5-BU treated cells x400

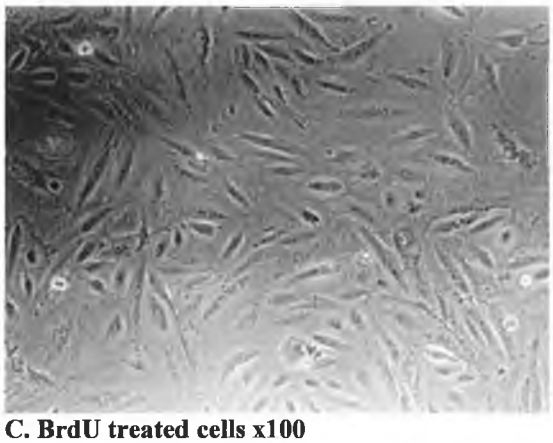
Figure 3.2.3 Continued. The effect of halogenated thymidine analogues on the cellular morphology of DLKP.



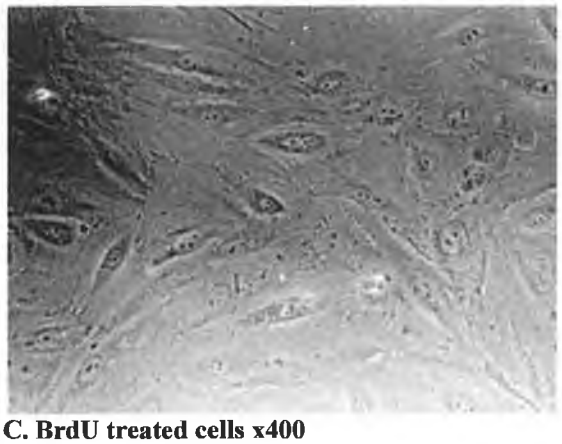
A. Untreated cells x100



B. Untreated cells x400

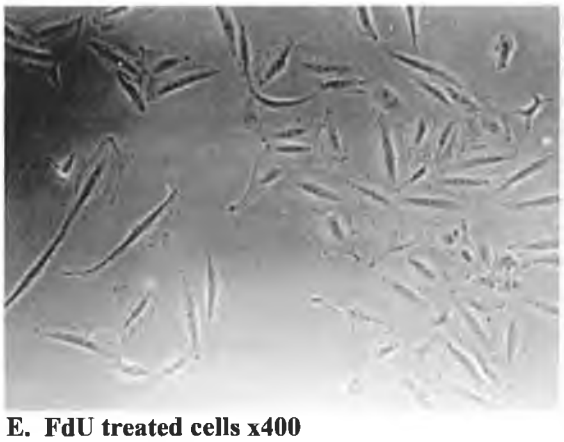


C. BrdU treated cells x100



C. BrdU treated cells x400

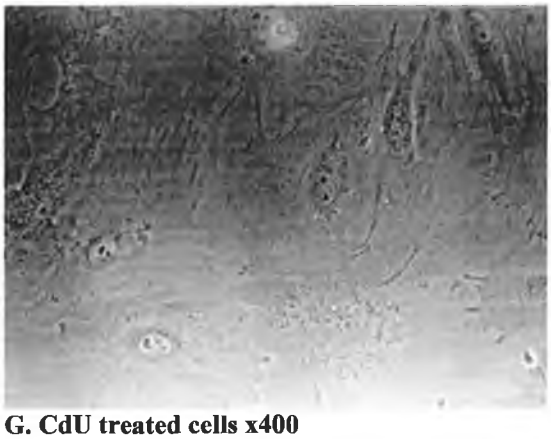
D. FdU treated cells x100



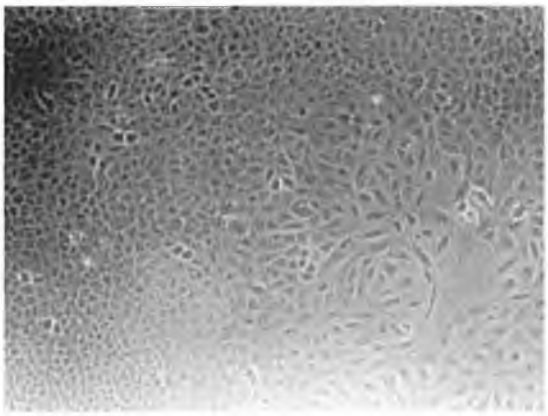
E. FdU treated cells x400

Figure 3.2.4 The effect of halogenated thymidine analogues on the cellular morphology of A549.

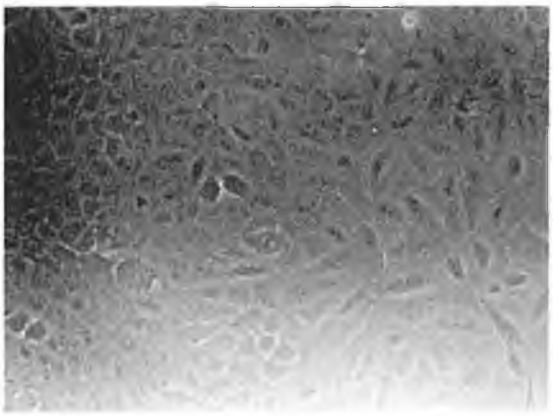
F. CdU treated cells x100



G. CdU treated cells x400



H. 5-BUr treated cells x100



I. 5-BUr treated cells x400

Figure 3.2.4 Continued. The effect of halogenated thymidine analogues on the cellular morphology of A549

3.2.2 ANALYSIS OF CHANGES IN MARKER PROTEIN EXPRESSION IN THYMIDINE ANALOGUE TREATED DLKP AND A549.

In both DLKP and A549 cells, exposure to 10μM BrdU was found to induce or increase expression of CK-8, CK-18, CK-19, β_1-integrin, and Ep-CAM (McBride *et al* 1999; Meleady and Clynes, manuscript submitted; results presented in this thesis). Using immunocytochemistry, western blot and RT-PCR analysis the expression of these proteins was investigated in DLKP and A549 cells exposed to CdU, 5,5'-FdU, and 5-BUr. A summary of the results obtained are presented in tables 3.2.2 and 3.2.3.

		TREATMENT PERFORMED				
		Control.	**BrdU Treated**	**5,5'-FdU Treated**	**CdU Treated**	**5-BU Treated**
CK-8	***Immunocytochemistry***	Negative	Induced Expression	Induced Expression	Induced Expression	Negative-No Change
	Western Blot	N/A	N/A	N/A	N/A	N/A
	RT-PCR		N/A	N/A	N/A	N/A
CK-18	***Immunocytochemistry***	Negative	Induced Expression	Induced Expression	Induced Expression	Negative-No Change
	Western Blot	N/A	N/A	N/A	N/A	N/A
	RT-PCR	N/A	N/A	N/A	N/A	N/A
CK-19	***Immunocytochemistry***	Negative	Induced Expression	Induced Expression	Induced Expression	Negative-No Change
	Western Blot	N/A	N/A	N/A	N/A	N/A
	RT-PCR	N/A	N/A	N/A	N/A	N/A
β1 Integrin	***Immunocytochemistry***	Slightly Positive	Induced Expression	Induced Expression	Induced Expression	Slightly Positive-No Change
	Western Blot	Positive	Induced Expression	N/A	N/A	N/A
	RT-PCR	Positive	Positive-No Change	N/A	N/A	N/A
Ep-CAM	***Immunocytochemistry***	Negative	Induced Expression	Induced Expression	Induced Expression	Negative-No Change
	Western Blot	Negative	Induced Expression	N/A	N/A	N/A
	RT-PCR	Positive	Positive-No Change	N/A	N/A	N/A

N/A = NOT AVAILABLE

Table 3.2.1 Summary of results obtained for different marker proteins in DLKP treated with various halogenated thymidine analogues.

		TREATMENT PERFORMED				
		Control.	**BrdU Treated**	**5,5'-FdU Treated**	**CdU Treated**	**5-BU Treated**
CK-8	***Immunocytochemistry***	Positive	Induced Expression	Induced Expression	Induced Expression	Positive-No Change (same as untreated)
	Western Blot	Positive	Induced Expression	Induced Expression	Induced expression	Positive-No Change (same as untreated)
	RT-PCR	Positive	Positive-No Change (same as untreated)	Positive-No Change (same as untreated)	Positive-No Change (same as untreated)	Positive-No Change (same as untreated)
CK-18	***Immunocytochemistry***	Positive	Induced Expression	Induced Expression	Induced Expression	Positive-No Change (same as untreated)
	Western Blot	Positive	Induced Expression	Induced Expression	Induced Expression	Positive-No Change (same as untreated)
	RT-PCR	Positive	Positive-No Change (same as untreated)	Positive-No Change (same as untreated)	Positive-No Change (same as untreated)	Positive-No Change (same as untreated)
CK-19	***Immunocytochemistry***	Positive	Induced Expression	Induced Expression	Unclear	Positive-No Change (same as untreated)
	Western Blot	Positive	Induced Expression	Induced Expression	Induced Expression	Positive-No Change (same as untreated)
	RT-PCR	Positive	Positive-No Change (same as untreated)	Positive-No Change (same as untreated)	Positive-No Change (same as untreated)	Positive-No Change (same as untreated)
β1 Integrin	***Immunocytochemistry***	Positive	Induced Expression	Down Regulation?	Induced Expression	Positive-No Change (same as untreated)
	Western Blot	Positive	Induced Expression	Induced Expression	Induced Expression	Positive-No Change (same as untreated)
	RT-PCR	Positive	N/A	N/A	N/A	N/A
Ep-CAM	***Immunocytochemistry***	Positive	Induced Expression	Down Regulation?	Induced Expression	Positive-No Change (same as untreated)
	Western Blot	Positive	Induced Expression	Induced Expression	Induced Expression	Positive-No Change (same as untreated)
	RT-PCR	Positive	Positive-No Change (same as untreated)	Positive-No Change (same as untreated)	Positive-No Changes (same as untreated)	Positive-No Change (same as untreated)

N/A = Not Available.

Table 3.2.2 Summary of results obtained for different marker proteins in A549 treated with various halogenated thymidine analogues.

3.2.2.1 Changes in cytokeratin-8 expression following treatment with halogenated thymidine analogues.

Immunocytochemical analysis of DLKP cells for expression of cytokeratin-8 (CK-8) showed induction by 2μM 5,5'-FdU (at a level greater than or equal to 10μM BrdU) (figure 3.2.5 C); and by 10μM CdU (at a level equal to 10μM BrdU) (figure 3.2.5 D). Treatment with 70μM 5-BUr did not however, induce any alteration (figure 3.2.5 E).

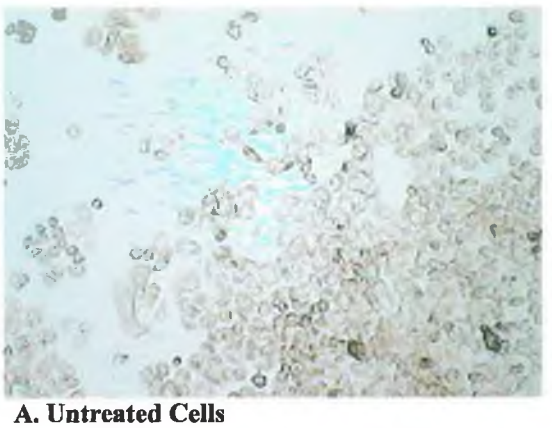
Immunocytochemical analysis of A549 treated with 2μM 5,5'-FdU showed induction of CK-8 expression (which appeared greater than 10μM BrdU) (figure 3.2.6 C). 10μM CdU treatment revealed enhanced expression for CK-8 protein (figure 3.2.6 D) (at a level comparable to 10μM BrdU); treatment with 70μM 5-BUr did not, however induce any alteration (figure 3.2.6 E).

In order to obtain a quantitative result, western blot analysis was conducted for CK-8 expression in A549 cells exposed to the halogenated thymidine analogues for up to 14 days (figure 3.2.7).

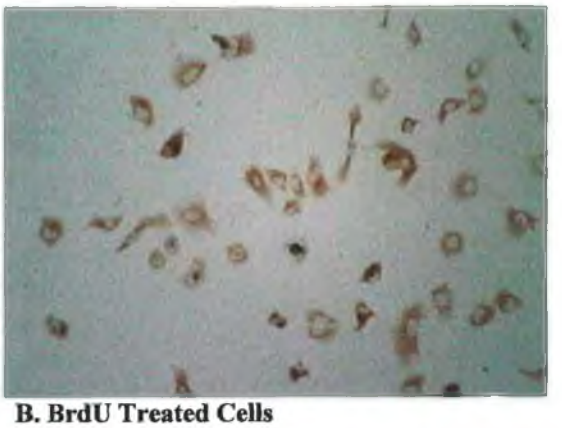
- It was previously shown in this laboratory that the level of CK-8 was enhanced over 14 days of treatment with BrdU (McBride *et al* 1999). However, such an induction was not observed in this experiment, though it was clearly visable with immunocytochemistry (figure 3.2.6 B).
- An increase in CK-8 protein was observed at day 7 and day 14 in 5,5'-FdU treated cells, this induction was greater than that observed for 10μM BrdU.
- 5-BU treatment appeared to decrease CK-8 substantially, this is followed by re-induction of CK-8 by day 14. Though no such change was visible with immunocytochemical techniques (figure 3.2.6 E).
- Treatment with 10μM CdU induced an increase in CK-8 expression. Unfortunately, due to the apparent cumulative cytotoxicity of CdU it was not possible to study changes in protein level over 14 days.

No change in the levels of CK-8 mRNA were detected by RT-RCR (figure 3.2.8) following 7-day treatment with 5,5'-FdU (2μM), BrdU (10μM), CdU (10μM) or 5-

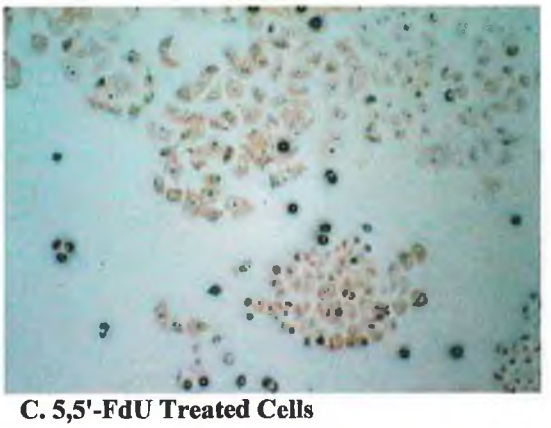
BUr (70μM). The main conclusion from this experiment is that the induction, at protein level observed for CK-8 following treatment with the base analogues must be at a post-transcriptional level.



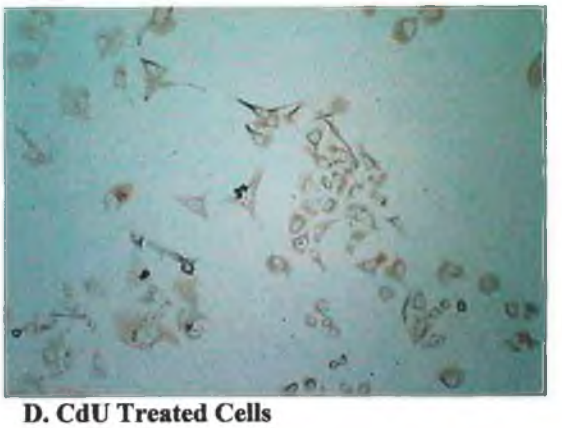
A. Untreated Cells



B. BrdU Treated Cells



C. 5,5'-FdU Treated Cells

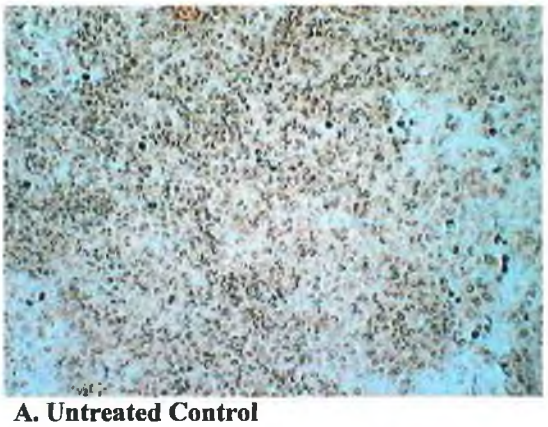


D. CdU Treated Cells

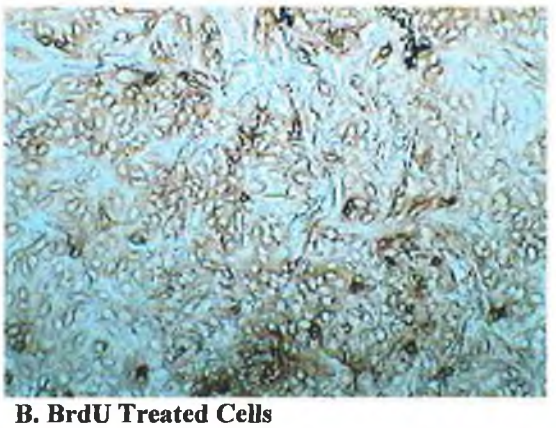
E. 5-BUr Treated Cells

F. Negative Control (No Primary Antibody)

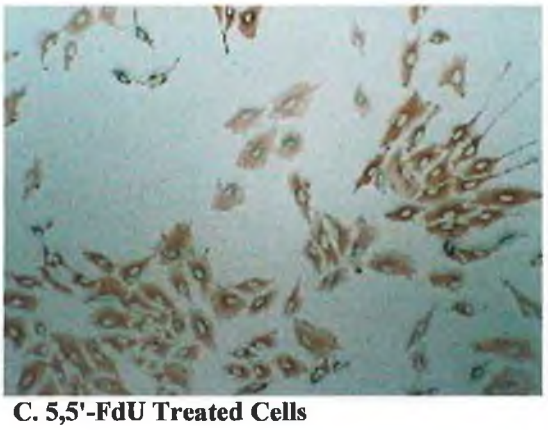
Figure 3.2.5 Immunocytochemcial analysis for cytokeratin-8 (CK-8) expression in DLKP cells (x100) treated with various halogenated thymidine analogues.



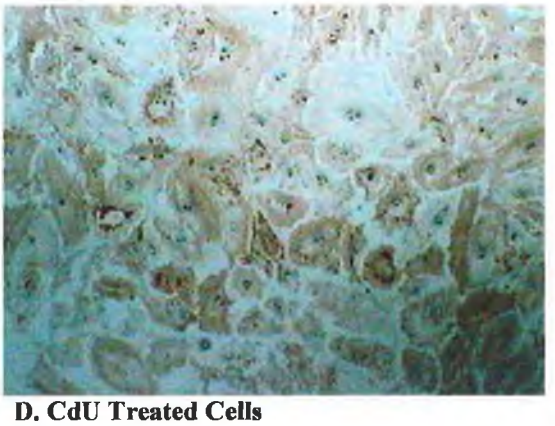
A. Untreated Control



B. BrdU Treated Cells



C. 5,5'-FdU Treated Cells



D. CdU Treated Cells

E. 5-BUr Treated Cells

F. Negative Control (No Primary Antibody)

Figure 3.2.6 Immunocytochemical analysis of cytokeratin-8 (CK-8) expression in A549 cells (x100) treated with various halogenated thymidine analogues.

Figure 3.2.7 **Western blot analysis for cytokeratin-8 (CK-8) expression in A549 cells following treatment with various halogenated thymidine analogues.**

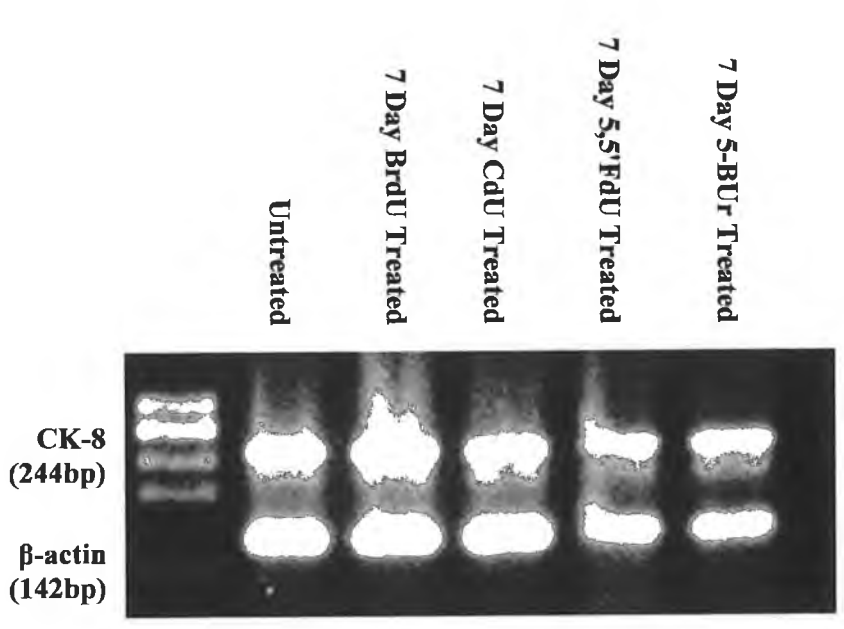


Figure 3.2.8 **RT-PCR analysis for cytokeratin-8 (CK-8) expression in A549 cells following treatment with various halogenated thymidine analogues.**

3.2.2.2 Changes in cytokeratin-18 expression following treatment with halogenated thymidine analogues.

Analysis by immunocytochemistry of DLKP cells treated with 2μM 5,5'-FdU, showed induced expression of cytokeratin-18 (CK-18) (figure 3.2.9 C), similar to that obtained following 10μM BrdU treatment (figure 3.2.9 B). A strong induction of CK-18 protein expression also occurred in 10μM CdU treated DLKP (figure 3.2.9 D) (at a level greater than or equal to 10μM BrdU); treatment with 70μM 5-BUr did not appear to alter CK-18 protein expression (figure 3.2.9 E).

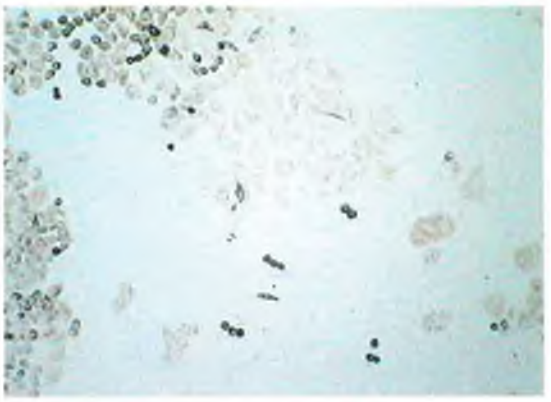
Immunocytochemical analysis of A549 treated with 2μM 5,5'-FdU showed enhanced expression for CK-18 (figure 3.2.10 C). The level of induced protein expression appears greater than enhanced expression following 10μM BrdU treatment (figure 3.2.10 B). Similarly, treatment with 10μM CdU revealed enhanced expression for CK-18 protein (figure 3.2.10 D), which appeared comparable to that observed in BrdU treatments. Accurate assessments were difficult due to the enlargement in morphology following 10μM CdU treatment. Exposure to 70μM 5-BUr did not appear to change protein expression levels (figure 3.2.10 E).

Western blot analysis was conducted for CK-18 expression in A549 cells treated with the halogenated thymidine analogues for up to 14 days to obtain a quantitative result (figure 3.2.11).

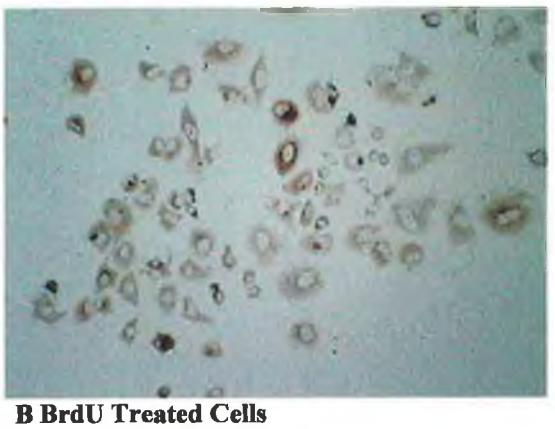
- It was previously shown in this laboratory the level of CK-18 was enhanced following treatment with BrdU (McBride *et al*, 1999). In the data presented here, this increase was not clearly seen.
- Treatment with 5,5'-FdU resulted in an induction of CK-18 expression in the day 7 and day 14 time points. The level of this increase was significantly larger then that observed for BrdU (figure 3.2.11).
- 10μM treatment of CdU for 7 days appeared to induce enhanced expression of CK-18. Unfortunately, due to the cumulative cytotoxicity associated with 10μM CdU treatment it was not possible to assess the change in CK-18 expression following 14 days of treatment.

- The level of CK-18 appeared to be decreased after 7 days of 5-BUr treatment, followed by a partial restoration by day 14. Due to time constraints it was not possible to investigate the validity of this result with fresh treatments at various concentrations of 5-BUr.

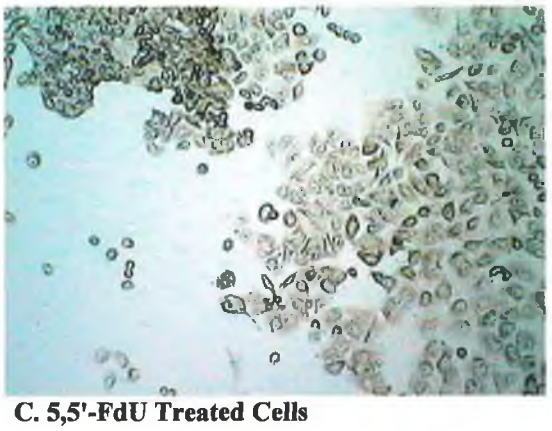
No change in the levels of CK-18 mRNA were detected by RT-RCR (figure 3.2.12) following 7-day treatment with 5,5'-FdU (2μM), BrdU (10μM), CdU (10μM) or 5-BUr (70μM). The main conclusion from this experiment is that the induction, at protein level observed for CK-18 following treatment with the base analogues must be at a post-transcriptional level.



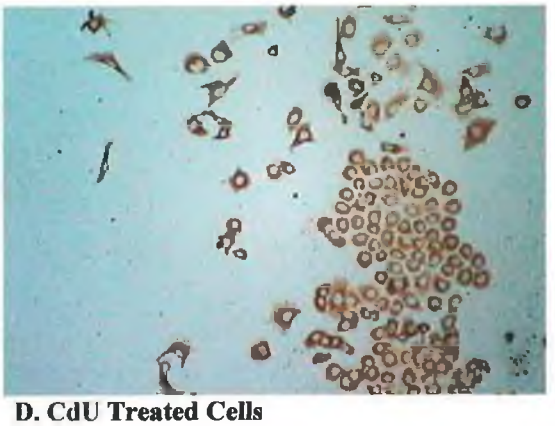
A. Untreated Cells



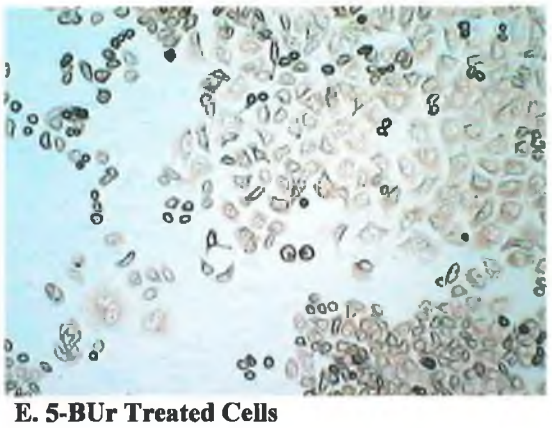
B BrdU Treated Cells



C. 5,5'-FdU Treated Cells



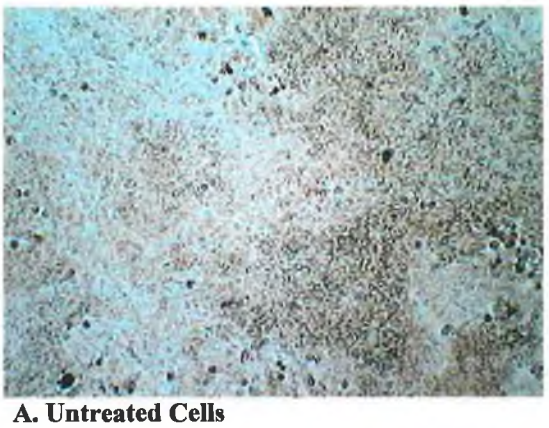
D. CdU Treated Cells



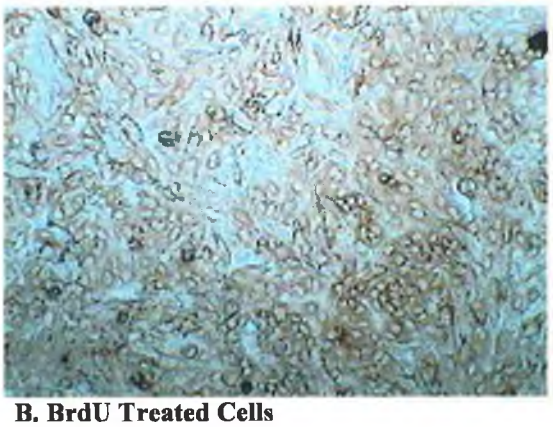
E. 5-BUr Treated Cells

F. Negative Control (No Primary Antibody)

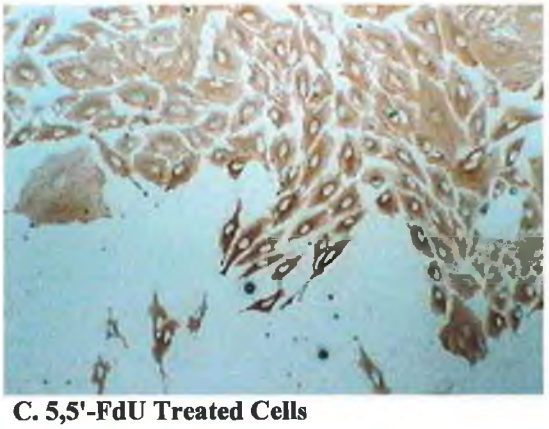
Figure 3.2.9 Immunocytochemical analysis of cytokeratin-18 (CK-18) expression in DLKP cells (x100) treated with various halogenated thymidine analogues.



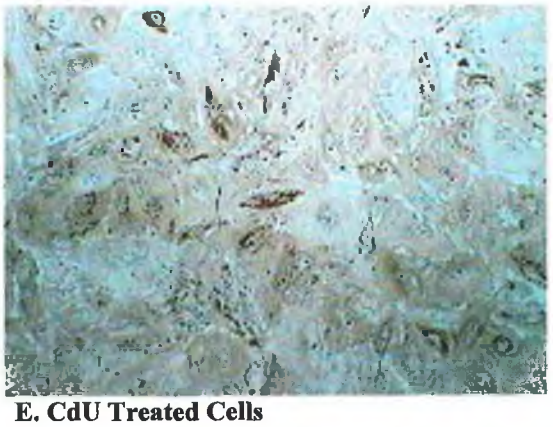
A. Untreated Cells



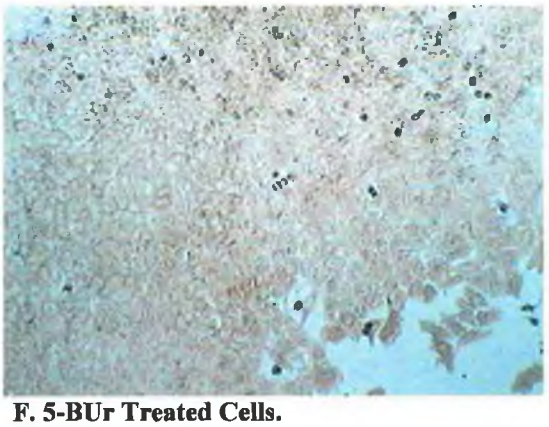
B. BrdU Treated Cells



C. 5,5'-FdU Treated Cells



E. CdU Treated Cells



F. 5-BUr Treated Cells.

F. Negative Control.(No Primary Antibody)

Figure 3.2.10 Immunocytochemical analysis of cytokeratin-18 (CK-18) expression in A549 cells (x100) treated with various halogenated thymidine analogues.

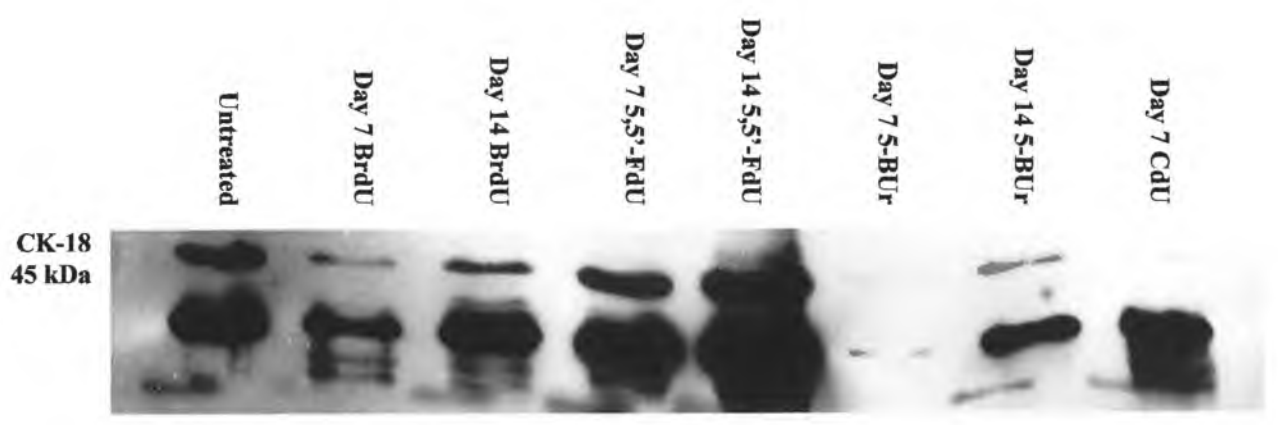


Figure 3.2.11 Western blot analysis for cytokeratin-18 (CK-18) expression in A549 cells following treatment with various halogenated thymidine analogues.

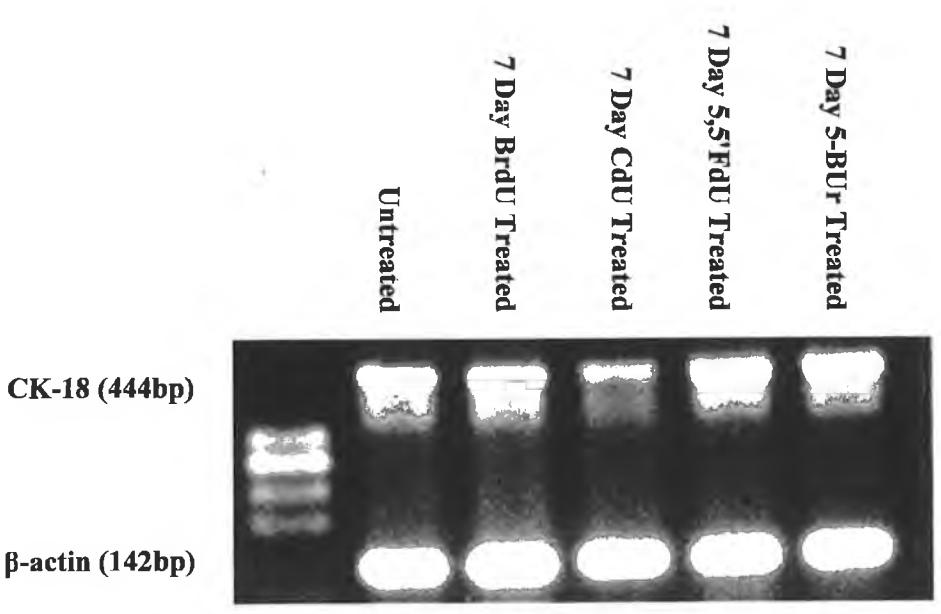


Figure 3.2.12 RT-PCR analysis for cytokeratin-18 (CK-18) expression in A549 cells following treatment with various halogenated thymidine analogues

3.2.2.3 Changes in cytokeratin-19 expression following treatment with halogenated thymidine analogues.

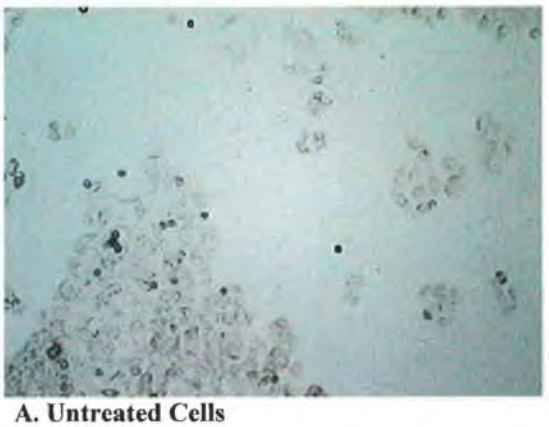
Immunocytochemical analysis of DLKP cells treated with 2μM 5,5'-FdU, showed the expression of cytokeratin-19 (CK-19) to be induced (figure 3.2.13 C). The intensity of the observed staining was comparable to that obtained following 10μM BrdU treatment (figure 3.2.13 B). Immunocytochemical analysis also suggested an induction of CK-19 protein expression in 10μM CdU treated cells (figure 3.2.13 D), (greater than that observed in 10μM BrdU treatment). In contrast immunocytochemistry treatment with 70μM 5-BUr did not exhibit any changes in CK-19 protein expression (figure 3.2.13 E).

Immunocytochemical analysis of A549 treated with 2μM 5,5'-FdU showed induction for cytokeratin-19 (CK-19) expression (figure 3.2.14 C), which appeared equal to the expression observed following 10μM BrdU treatment (figure 3.2.14 B). Immunocytochemistry of 10μM CdU treated cells revealed enhanced expression for CK-8 protein (figure 3.2.14 D) (comparable to that observed in BrdU treatment). No change in CK-19 protein expression occurred, in A549 treated with 70μM 5-BUr (figure 3.2.14 E).

Western blot analysis for CK-19 expression in A549, following treatment with the halogenated thymidine analogues, was conducted (figure 3.2.15).

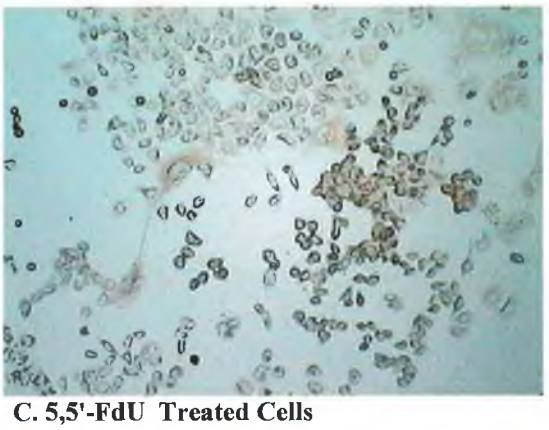
- In agreement with previous experiments the level of CK-19 was enhanced at the day 7 and day 14 time points following treatment with BrdU (Meleady *et al*, manuscript in preparation).
- In cells treated with 5,5'-FdU, an increase in CK-19 expression was observered at the day 7-time point. The day 14-time point of 5,5'-FdU treatment produced a substantial increase in CK-19 expression.
- 5-BUr treated cells the expression of CK-19 appeared unchanged at the day 7 and day 14 time points.
- Treatment with 10μM CdU appears to show CK-19 expression at the day-7 time point.

The effect of the halogenated thymidine analogues on CK-19 transcription rates in A549 was investigated with RT-PCR analysis (figure 3.2.16). Treatment by 5,5'-FdU (2μM) for 7 and 14 days did not produce any obvious alteration in the mRNA levels in A549. Similarly the levels if mRNA in A549 cells treated with CdU (10μM) or 5-BUr (70μM) over 7 and 14 days did not alter mRNA levels of CK-19. Exposure to 5-BUr did not appear to alter the mRNA level at the day 7 and day 14 time points.

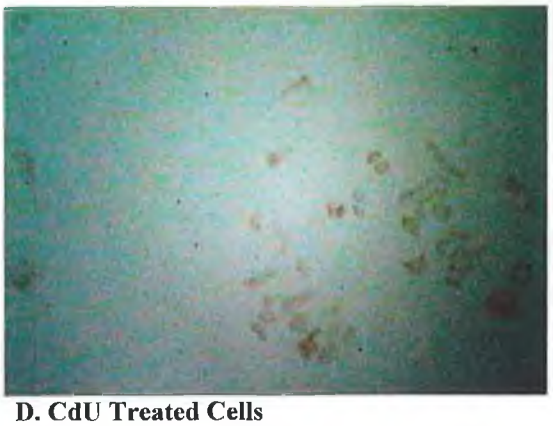


A. Untreated Cells

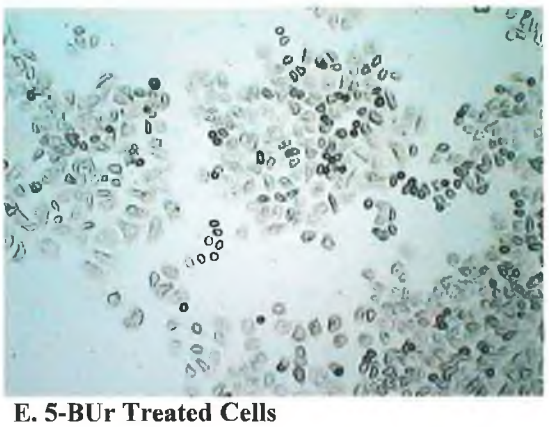
B. BrdU Treated Cells



C. 5,5'-FdU Treated Cells



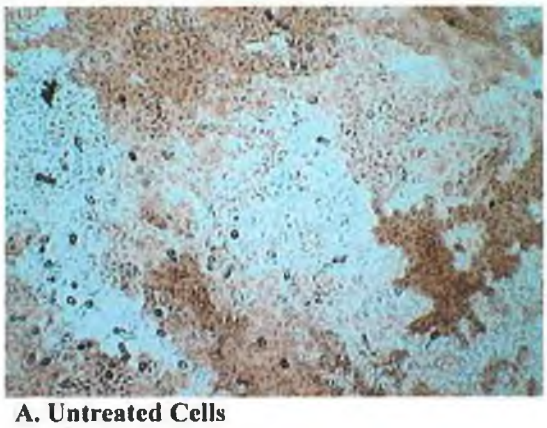
D. CdU Treated Cells



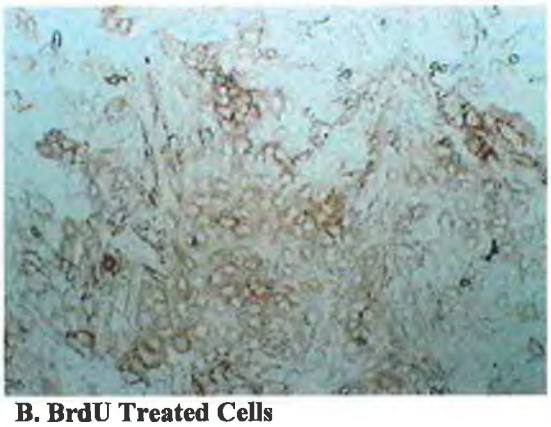
E. 5-BUr Treated Cells

F. Negative Control (No Primary Antibody)

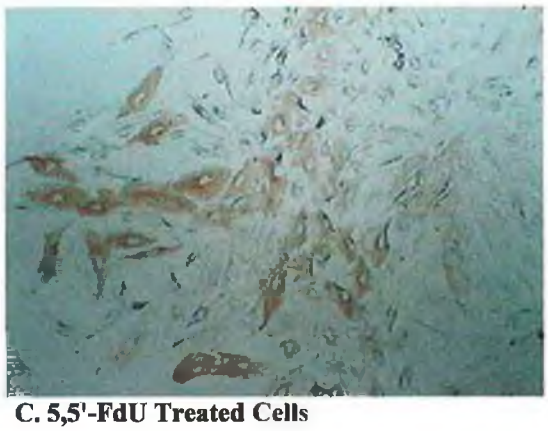
Figure 3.2.13 Immunocytochemical analysis for cytokeratin-19 (CK-19) in DLKP cells (x100) treated with various halogenated thymidine analogues.



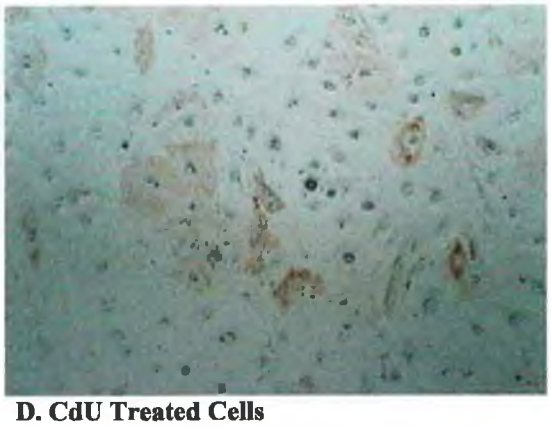
A. Untreated Cells



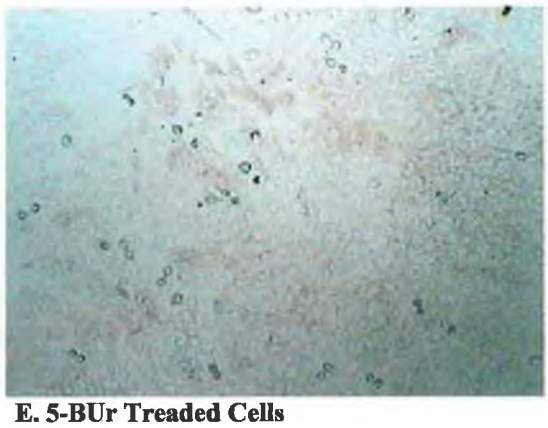
B. BrdU Treated Cells



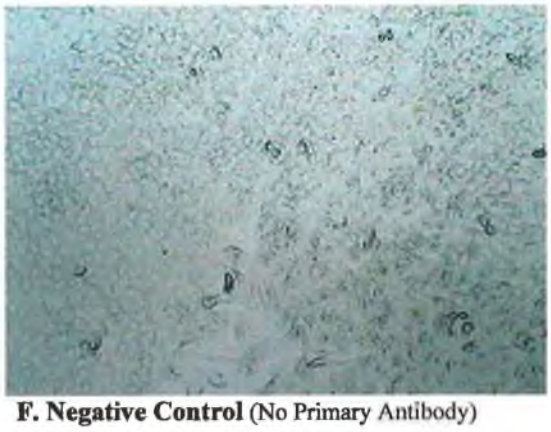
C. 5,5'-FdU Treated Cells



D. CdU Treated Cells



E. 5-BUr Treaded Cells



F. Negative Control (No Primary Antibody)

Figure 3.2.14 Immunocytochemical analysis for cytokeratin-19 (CK-19) expression in A549 cells (x100) treated with various halogenated thymidine analogues.

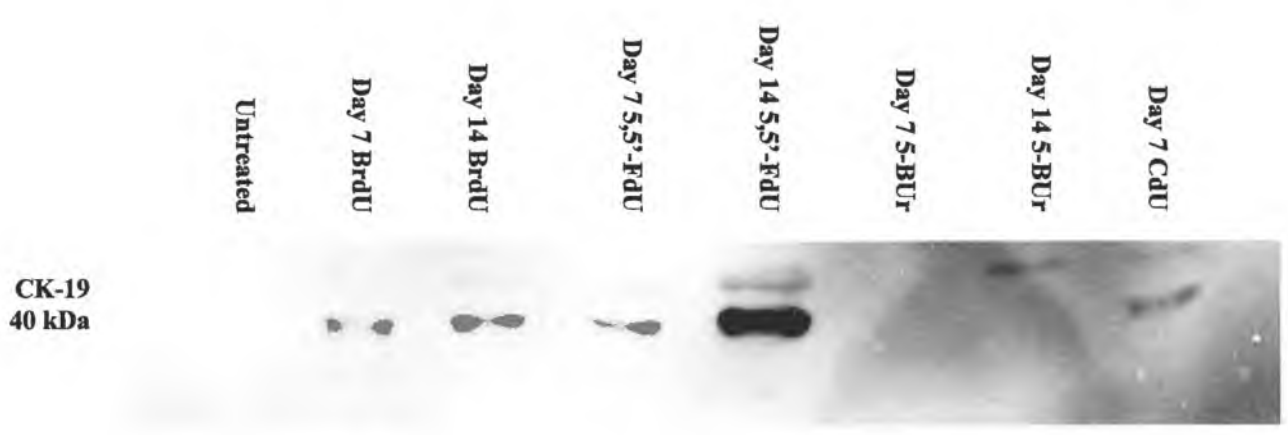


Figure 3.2.15 Western blot analysis of cytokeratin-19 (CK-19) expression in A549 cells following treatment with various thymidine analogues.

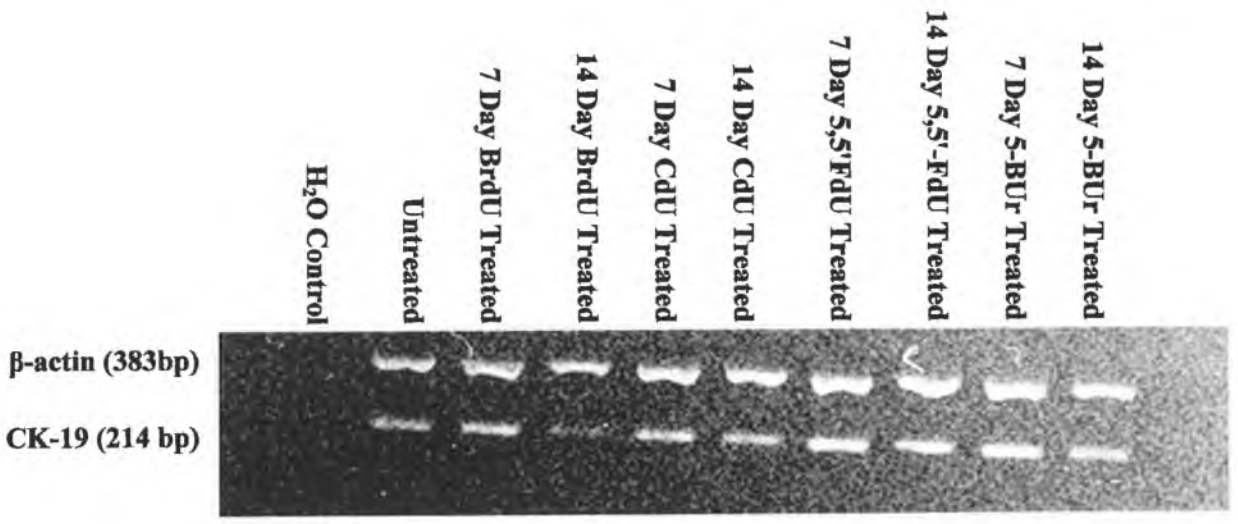


Figure 3.2.16 RT-PCR analysis for cytokeratin-19 (CK-19) expression in A549 cells following treatment with various halogenated thymidine analogues

3.2.2.4 Changes in Ep-CAM expression following treatment with halogenated thymidine analogues.

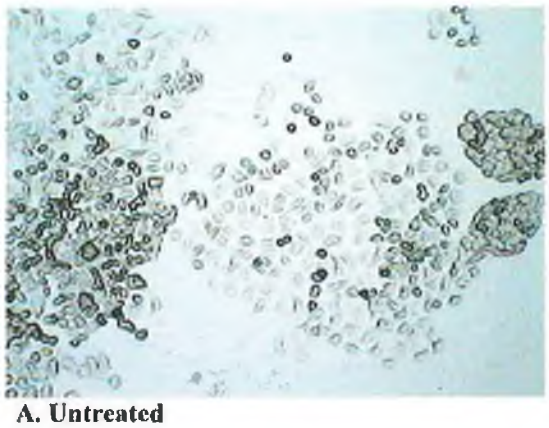
Analysis by immunocytochemistry of DLKP treated with 5,5'-FdU revealed an apparent induction of Ep-CAM expression (figure 3.2.17 C). Similarly CdU treatment induced an expression of Ep-CAM (figure 3.2.17 D) (that was equal to or greater than that observed following BrdU treatment). Immunocytochemical analysis did not reveal an induction of Ep-CAM in 70μM 5-BUr treated cells (figure 3.2.17 E).

A549 treated with 5,5'-FdU revealed an apparent reduction of Ep-CAM expression by immunocytochemistry analysis (figure 3.2.18 C), in contrast with 10μM BrdU treatment, which induces Ep-CAM expression (figure 3.2.18 B). 10μM CdU treatment induced an expression of Ep-CAM (figure 3.2.18 D) (at least equal to that of 10μM BrdU). Immunocytochemical analysis did not reveal an induction of Ep-CAM in 70μM 5-BUr treated cells (figure 3.2.18 E).

Figure 3.2.19 shows the results of western blot analysis of A549 treated with halogenated thymidine analogues.

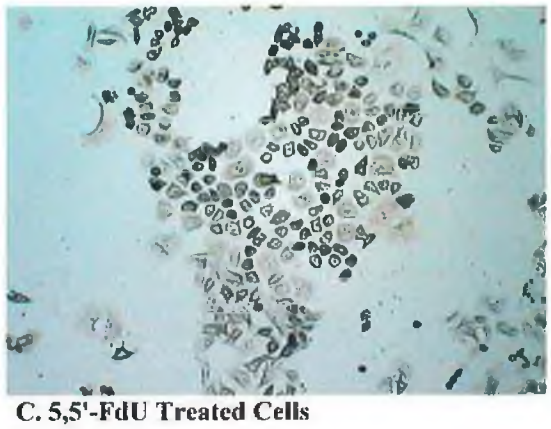
- In agreement with earlier experiments (section 3.1.2) the treatment with 10μM BrdU induced Ep-CAM expression at 7 and 14 days.
- Exposure to 5,5'-FdU (2μM) for 7 days induced Ep-CAM expression, Ep-CAM expression was further induced after 14 days of treatment, and the level of induction appeared to be greater than for BrdU. This result contradicts the observations made by immunocytochemistry, which showed a decrease in Ep-CAM expression. Unfortunately due to time constraints repeat experiments with fresh treatments was not possible.
- In 5-BUr treated cells there was an apparent induction of Ep-CAM in the day 7 and day 14 time points. It is an interesting feature, since no induction of cytokeratin-8, -18, or -19 was observed with 5-BUr treatment.
- Treatment with CdU did appear to induce Ep-CAM slightly over 7 days.

The effect of the halogenated thymidine analogues on Ep-CAM mRNA expression was investigated with RT-PCR analysis (figure 3.2.20). Treatment with (10μM) CdU and (70μM) 5-BUr for 7 days did not appear to alter the mRNA level of Ep-CAM in A549. Treatment with 5,5'-FdU appeared to slightly decrease the Ep-CAM mRNA level. Since an increase in protein expression was observed in immunocytochemistry and western blot analysis, the validity of this result is questionable and may be due to sample degradation.

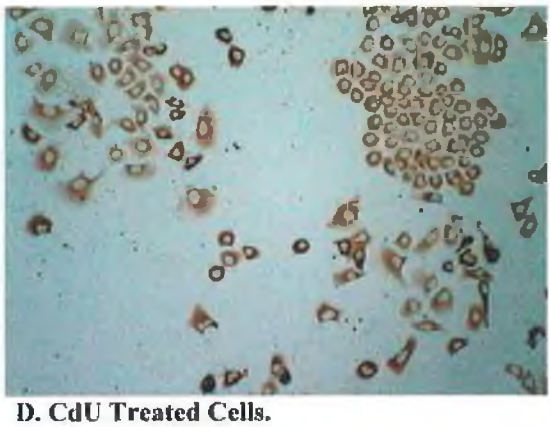


A. Untreated

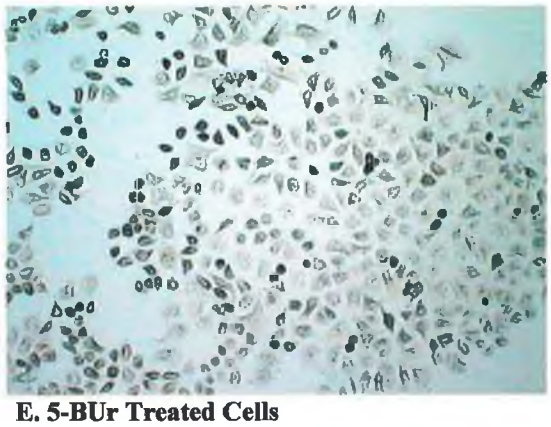
B. BrdU Treated Cells



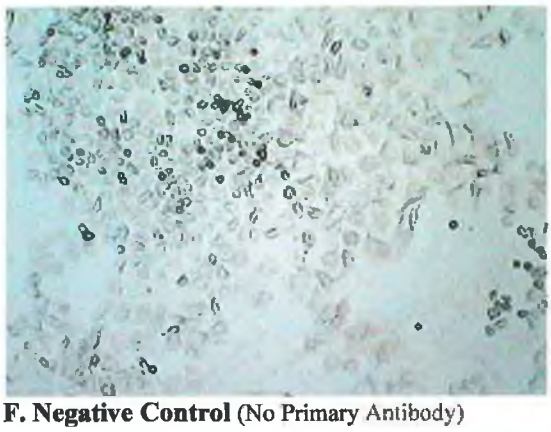
C. 5,5'-FdU Treated Cells



D. CdU Treated Cells.

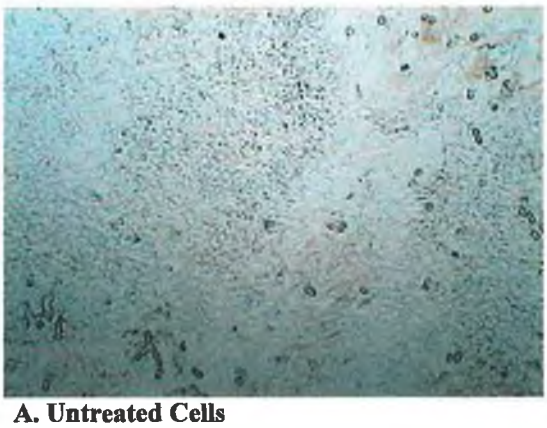


E. 5-BUr Treated Cells



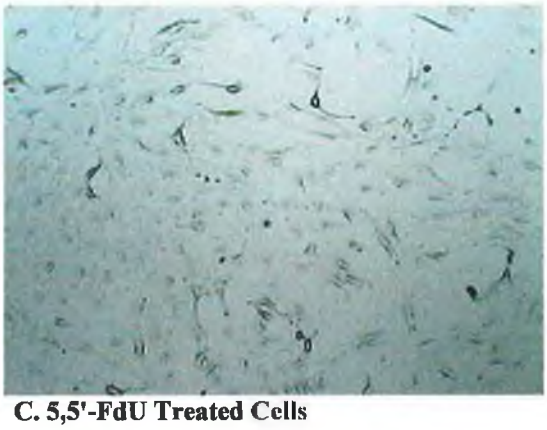
F. Negative Control (No Primary Antibody)

Figure 3.2.17 Immunocytochemical analysis for Ep-CAM expression in DLKP cells (x100) treated with various halogenated thymidine analogues.



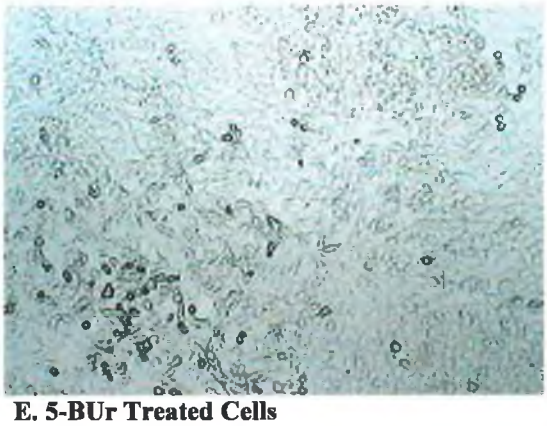
A. Untreated Cells

B. BrdU Treated Cells

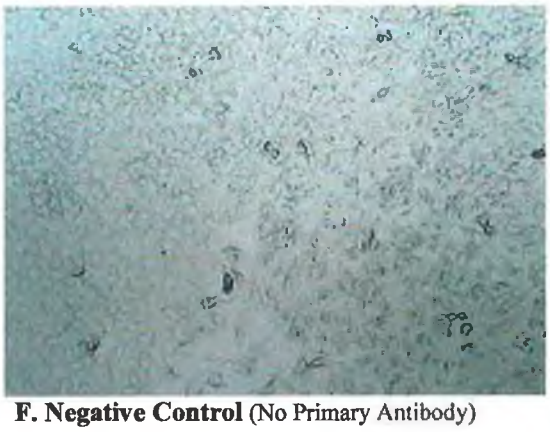


C. 5,5'-FdU Treated Cells

D. CdU Treated Cells



E. 5-BUr Treated Cells



F. Negative Control (No Primary Antibody)

Figure 3.2.18 Immunocytochemical analysis for Ep-CAM expression in A549 cells (x100) treated with various halogenated thymidine analogues.

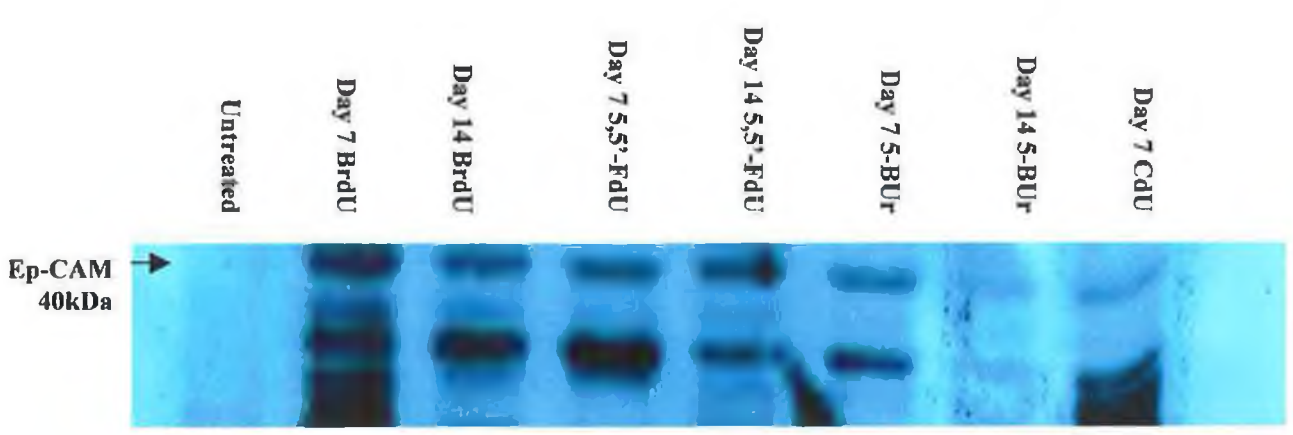


Figure 3.2.19 Western blot analysis of Ep-CAM expression in A549 cells following treatment with various thymidine analogues.

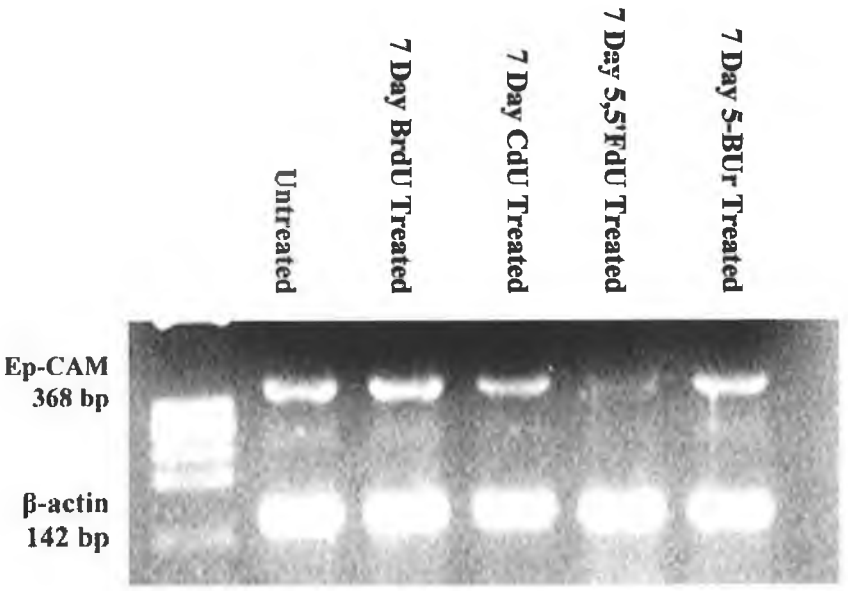


Figure 3.2.20 RT-PCR analysis for Ep-CAM expression in A549 cells following treatment with various halogenated thymidine analogues.

3.2.2.5 Changes in β_1 integrin expression following treatment with halogenated thymidine analogues.

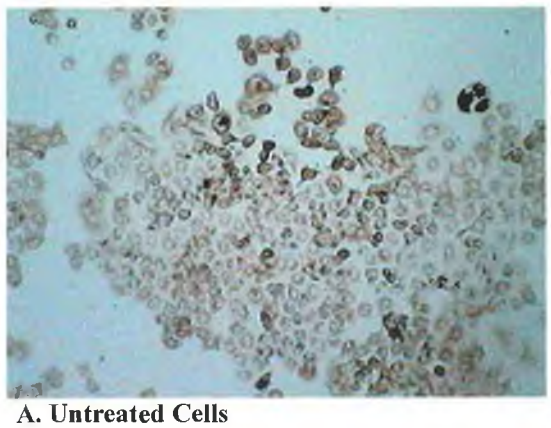
Immunocytochemical analysis of DLKP cells treated with 2μM 5,5'-FdU, showed induced expression of β_1 integrin (figure 3.2.21 C), which was equivalent to that observed for 10μM BrdU (figure 3.2.21 B). An induction for β_1 integrin expression was also observed in 10μM CdU treatments (figure 3.2.21 D) (equal to 10μM BrdU treatment), in contrast 70μM 5-BUr did not exhibit any changes in β_1 integrin protein expression (figure 3.2.21 E).

Analysis of A549 treated with 2μM 5,5'-FdU by immunocytochemical methods showed reduced expression for β_1-integrin (figure 3.2.22 C) (less then untreated A549 cells (figure 3.2.22 A)). Induced expression for β_1 integrin protein with 10μM CdU treatment (figure 3.2.22 D) (comparable to BrdU treatment); no change in protein expression appeared to occur in 70μM 5-BUr treatment (figure 3.2.22 E).

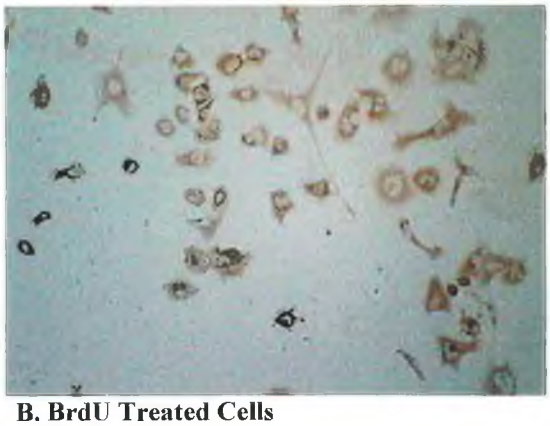
In order to obtain a quantitative result western blot analysis was conducted for β_1 integrin expression in A549 cells exposed to the halogenated thymidine analogues for up to 14 days (figure 3.2.23).

- As shown previously in this laboratory the level of β_1 integrin expression was enhanced at day 7 and day 14 following treatment with BrdU (Meleady *et al.*, manuscript in preparation).
- Induction of β_1 integrin protein occurred over the 14 days of 5,5'-FdU treatment, which was greater then the increase observed for BrdU treatment. It also calls into doubt the validity of the immunocytochemical result (figure 3.2.23 C), which showed inhibition of β_1-integrin expression. Unfortunately due to time constraints it was not possible to conduct repeat experiments necessary for further investigation.
- 5-BUr treatment may have caused a slight induction in β_1 integrin expression.
- Treatment with 10μM CdU over 7 days induced a slight increase in β_1 integrin protein expression. due to the difficulty of culturing enough cells for the assay it

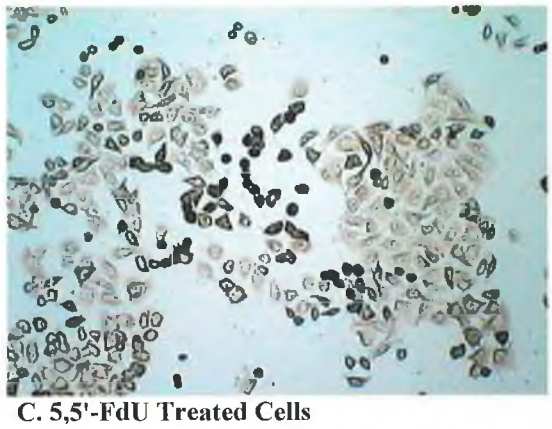
was not possible to investigate an increase in β_1 integrin protein expression beyond 7 days.



A. Untreated Cells

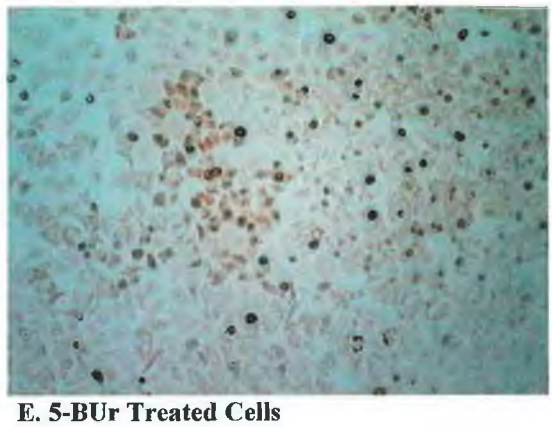


B. BrdU Treated Cells



C. 5,5'-FdU Treated Cells

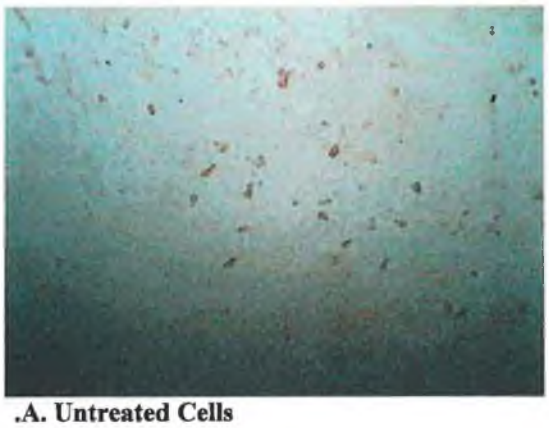
D. CdU Treated Cells



E. 5-BUr Treated Cells

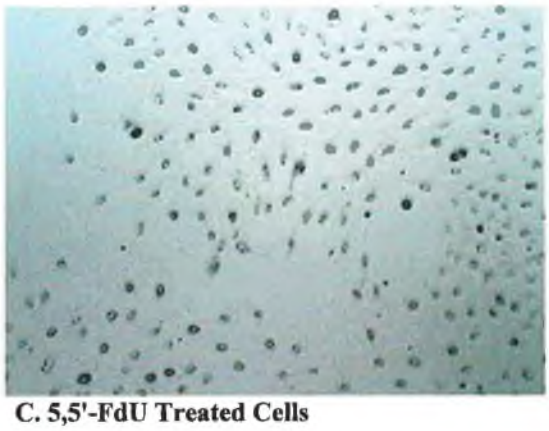
F. Negative Control (No Primary Antibody)

Figure 3.2.21 Immunocytochemical analysis for β1-integrin expression in DLKP cells (x100) treated with various halogenated thymidine analogues.

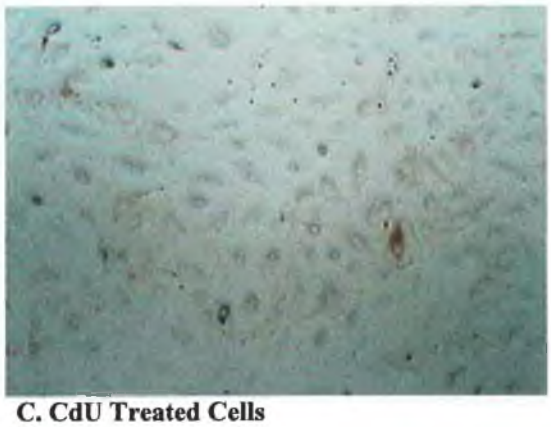


.A. Untreated Cells

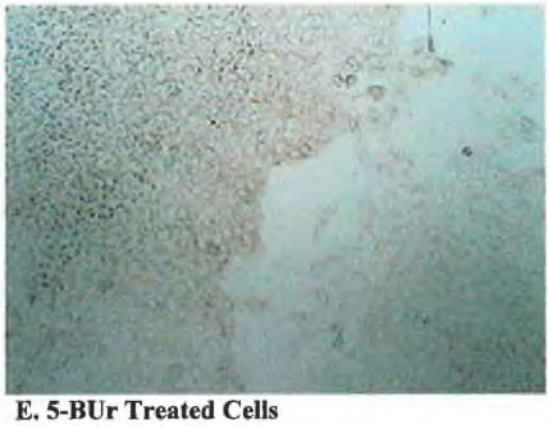
B. BrdU Treated Cells



C. 5,5'-FdU Treated Cells



C. CdU Treated Cells



E. 5-BUr Treated Cells

F. Negative Control (No Primary Antibody)

Figure 3.2.22 Immunocytochemistry analysis for β1 Integrin expression in A549 cells (x100) treated with various halogenated thymidine analogues.

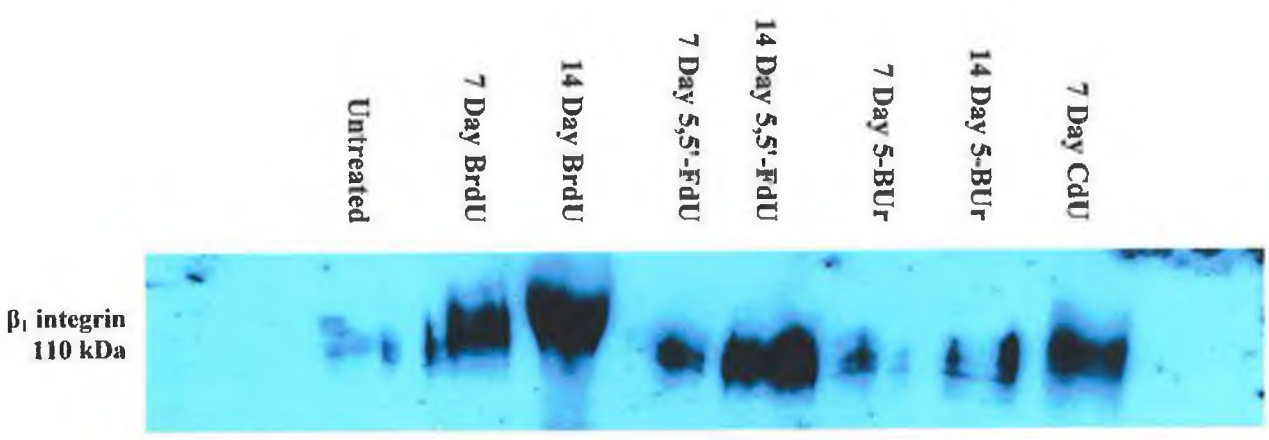


Figure 3.2.23 Western blot analysis of β_1-integrin expression in A549 cells following treatment with various thymidine analogues.

3.3 INVESTIGATION INTO THE ABILITY OF A COMPLEX HORMONE SUPPLEMENTED MEDIA TO INDUCE DIFFERENTIATION IN THE LUNG CELL LINES DLKP AND A549.

Previous reports from Prof. Emura's laboratory in Hannover had shown the fetal hamster lung cell line M3E3/C3 had properties suggestive of a possible lung stem cell e.g. it is, like DLKP, cytokeratin negative. The use of a hormone supplemented medium caused these cells to differentiate *in vitro* and to acquire a type II pneumocyte-like phenotype (Germann *et al*, 1993).

The culture medium was adapted for the DLKP and A549 cell line models by eliminating the soft agar overlay and reducing the serum concentration to 5%.

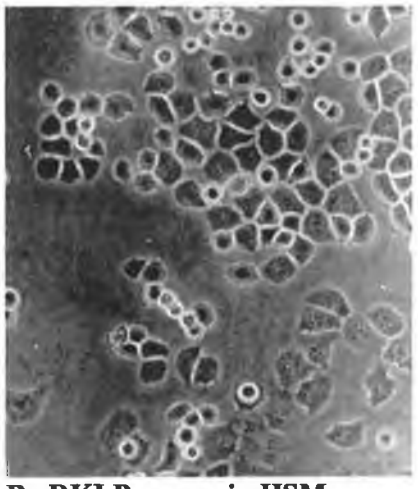
Since the medium contained several biologically active compounds, it was proposed that this study would allow an assessment of alternative *in vitro* induced differentiation pathways which might or might not be similar with the pathway(s) induced by the halogenated thymidine analogues. to be made of the physiological relevance of BrdU induced differentiation.

3.3.1 MORPHOLOGICAL ANALYSIS.

The lung cell lines were grown for 7 days in the hormone supplemented medium (HSM) and any change in morphology was noted. The DLKP cells obtained a more squamous morphology in HSM (figure 3.3.1 B) compared to cells in normal medium (figure 3.3.1 A).

The cell line A549, which is often used as a model for type II pneumocytes, also developed a squamous morphology and an increase in the number of cytoplasmic granules also appeared to occur (figure 3.3.2 B) when compared to cells grown in normal media (figure 3.3.2 A).

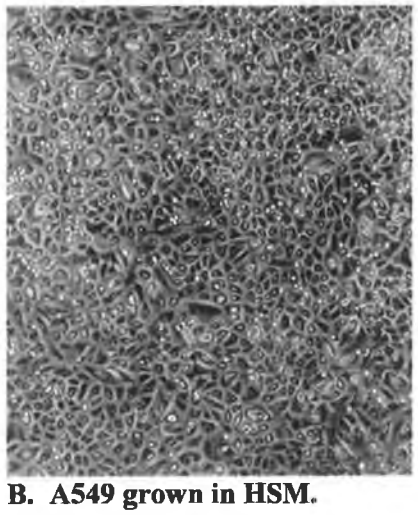
A. DLKP Untreated.



B. DKLP grown in HSM.

Figure 3.3.1 Morphology change in DLKP following growth in HSM (x100).

A. A549 Untreated.



B. A549 grown in HSM.

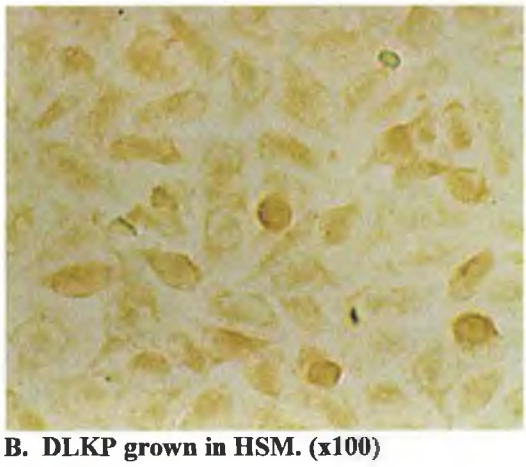
Figure 3.3.2 Morphology change in A549 following growth in HSM (x100).

3.3.2 IMMUNOCYTOCHEMICAL ANALYSIS OF MARKER PROTEINS IN DLKP AND A549 GROWN IN HSM.

Immunocytochemical analysis for CK-8 expression in DLKP following growth in HSM showed an induction of CK-8 expression (figure 3.3.3). Similarly, cytokeratin-18 (figure 3.3.5) and cytokeratin-19 (figure 3.3.7) showed induction by HSM. Immunocytochemistry for Ep-CAM showed strong induction following growth in HSM (figure 3.3.9).

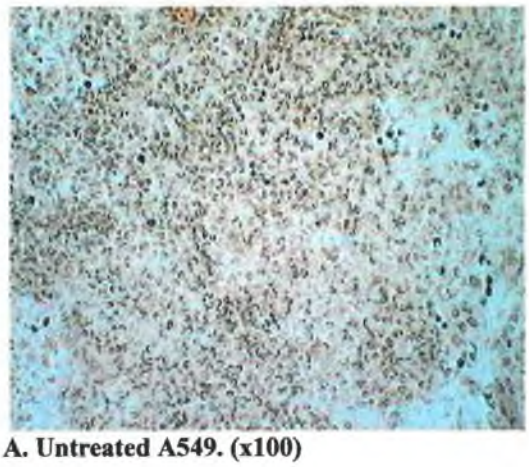
According to immunocytochemical analysis, A549 cells appeared to show a slight induction of cytokeratin-8 expression following growth in HSM (figure 3.3.4), cytokeratins –18 (figure 3.3.6) and –19 (figure 3.3.8) also appeared to be induced following growth in HSM. The expression of Ep-CAM was induced following growth of A549 in HSM (figure 3.3.10).

A. Untreated DLKP. (x100)

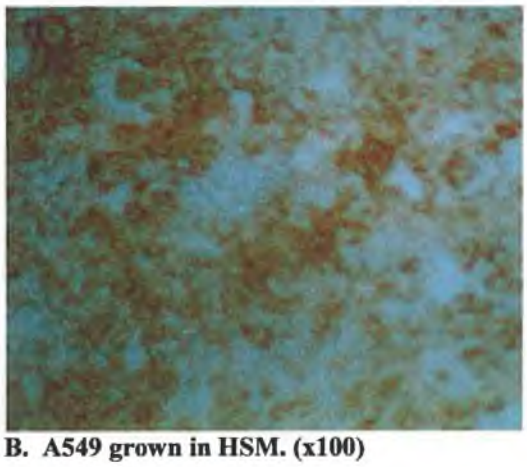


B. DLKP grown in HSM. (x100)

Figure 3.3.3 Immunocytochemistry for CK-8 expression in DLKP grown in HSM.

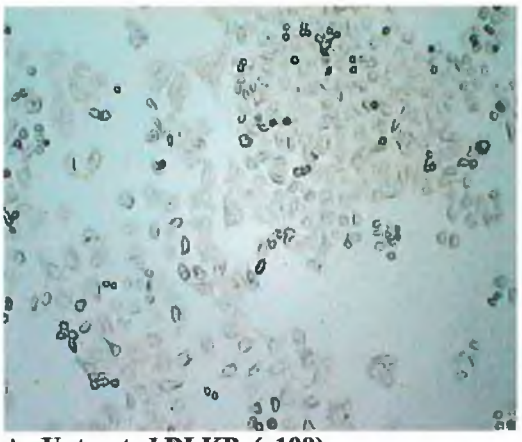


A. Untreated A549. (x100)

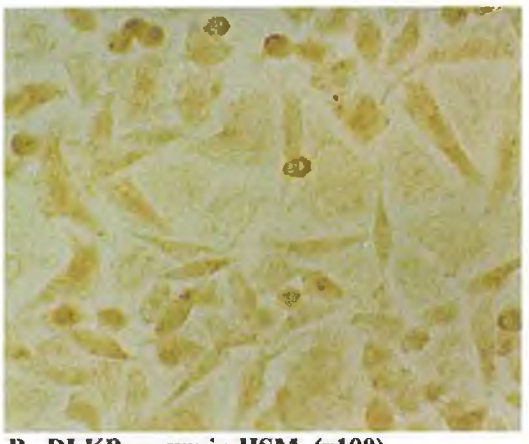


B. A549 grown in HSM. (x100)

Figure 3.3.4 Immunocytochemistry for CK-8 expression in A549 grown in HSM.

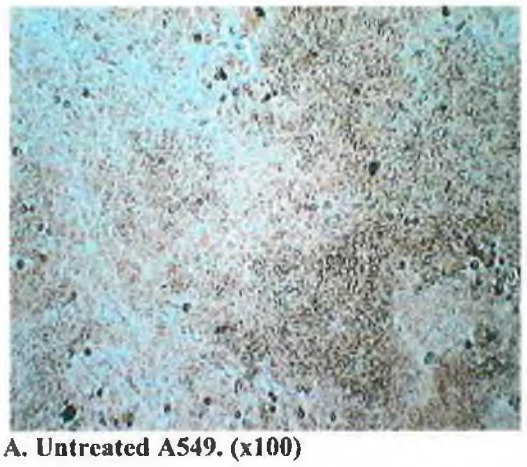


A. Untreated DLKP. (x100)

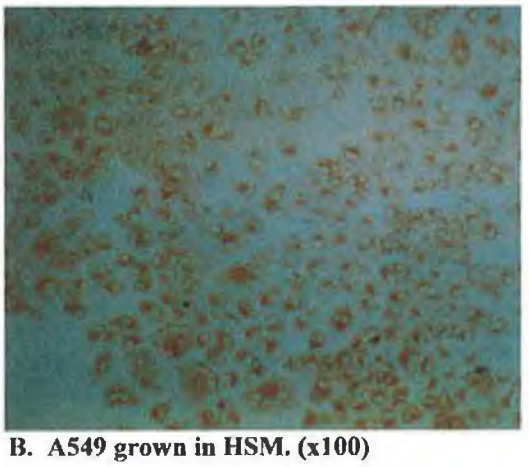


B. DLKP grown in HSM. (x100)

Figure 3.3.5 Immunocytochemistry for CK-18 expression in DLKP grown in HSM.

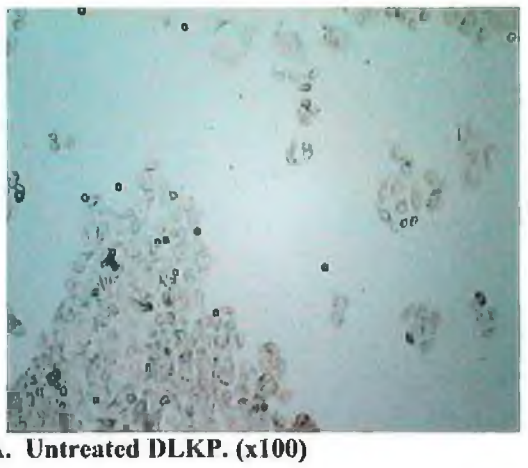


A. Untreated A549. (x100)

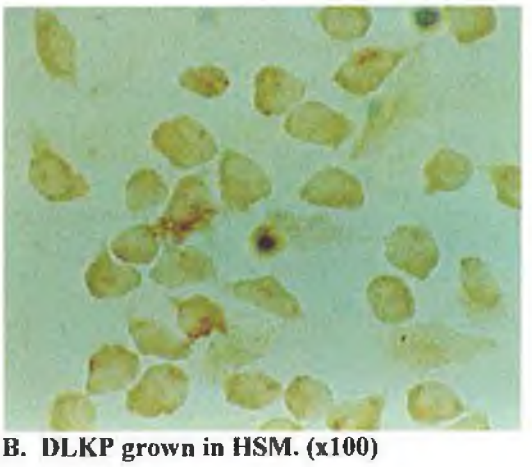


B. A549 grown in HSM. (x100)

Figure 3.3.6 immunocytochemistry for CK-18 expression in A549 grown in HSM.

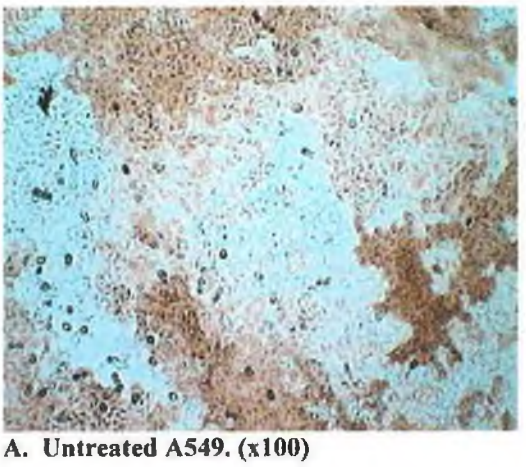


A. Untreated DLKP. (x100)



B. DLKP grown in HSM. (x100)

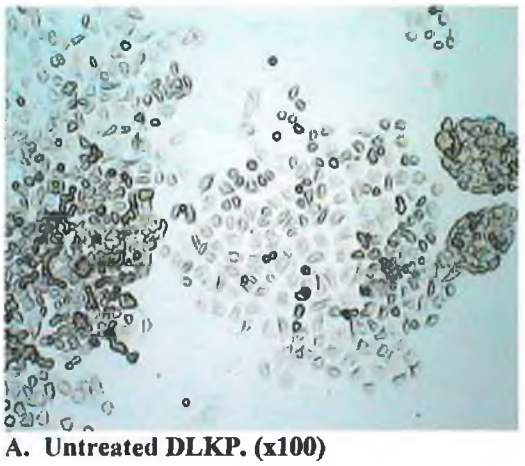
Figure 3.3.7 Immunocytochemistry for CK-19 expression in DLKP grown in HSM.



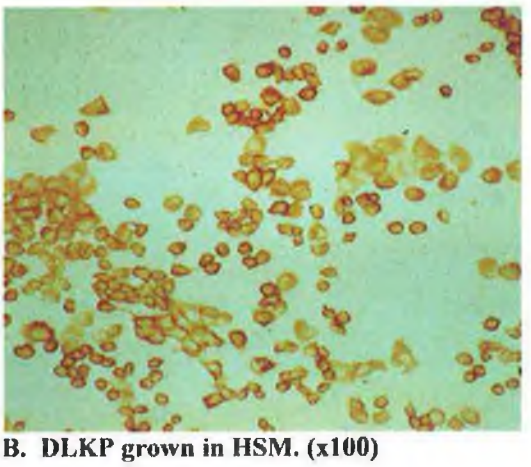
A. Untreated A549. (x100)

B. A549 grown in HSM. (x100)

Figure 3.3.8 Immunocytochemistry for CK-19 expression in A549 grown in HSM.

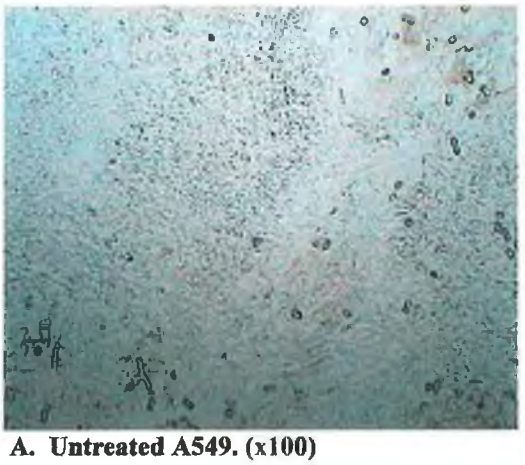


A. Untreated DLKP. (x100)

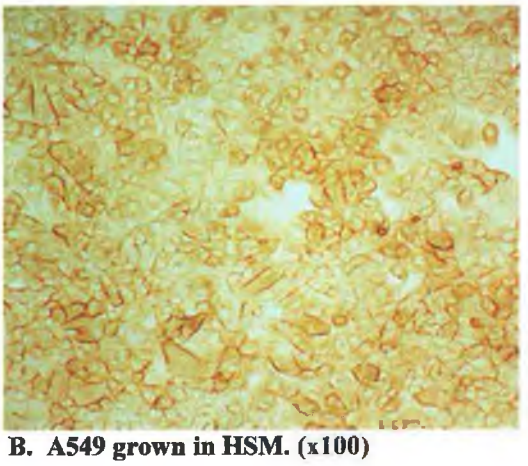


B. DLKP grown in HSM. (x100)

Figure 3.3.9 Immunocytochemistry for Ep-CAM expression in DLKP grown in HSM.



A. Untreated A549. (x100)



B. A549 grown in HSM. (x100)

Figure 3.3.10 Immunocytochemistry for Ep-CAM expression in A549 grown in HSM.

3.3.3 WESTERN BLOT ANALYSIS OF MARKER PROTEINS IN DLKP AND A549 GROWN IN HSM.

To quantify the changes observed in the immunocytochemical analysis (section 3.3.2), western blot analysis was performed on DLKP and A549 cells that had been treated with HSM for 7 and 14 days.

- No detectable CK-8, CK-18, and CK-19 was observed in DLKP grown in HSM, suggesting that immunoprecipitation was required (figure 3.3.11, 3.3.12, 3.3.13).
- An induction in Ep-CAM expression was observed in DLKP grown in HSM for 21 days (figure 3.3.14) confirming the result obtained by immunocytochemistry.

- In A549 no obvious increase was observed for CK-8 when they were grown in HSM (figure 3.3.11).
- There was an increase in CK-18 expression in A549 grown in HSM (figure 3.3.12).
- CK-19 expression increased in the A549 cells cultured in HSM over 14 days, thus confirming the results that were obtained by immunocytochemistry (figure 3.3.13).
- Ep-CAM expression increased in A549 cultured in HSM for 21 days (figure 3.3.14)

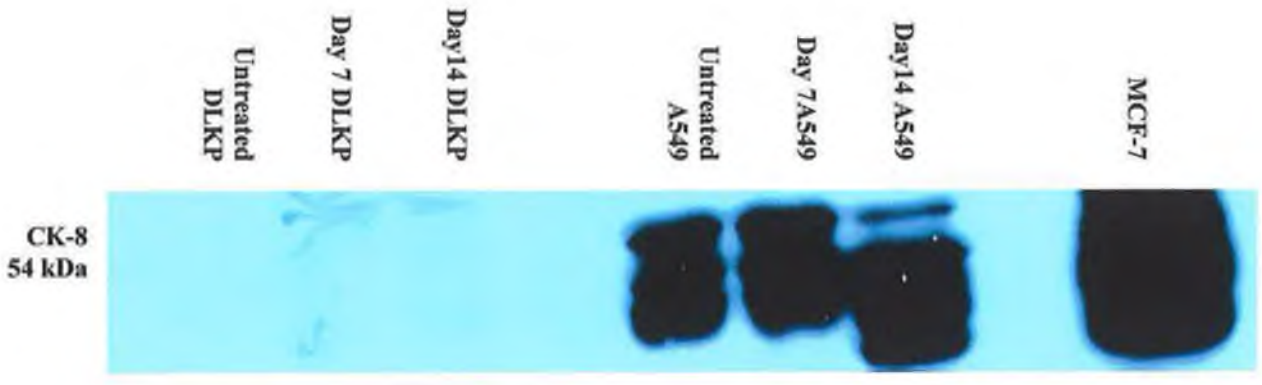


Figure 3.3.11 Western Blot analysis of CK 8 expression in DLKP and A549 grown in HSM.

Figure 3.3.12 Western Blot analysis of CK 18 expression in DLKP and A549 grown in HSM.

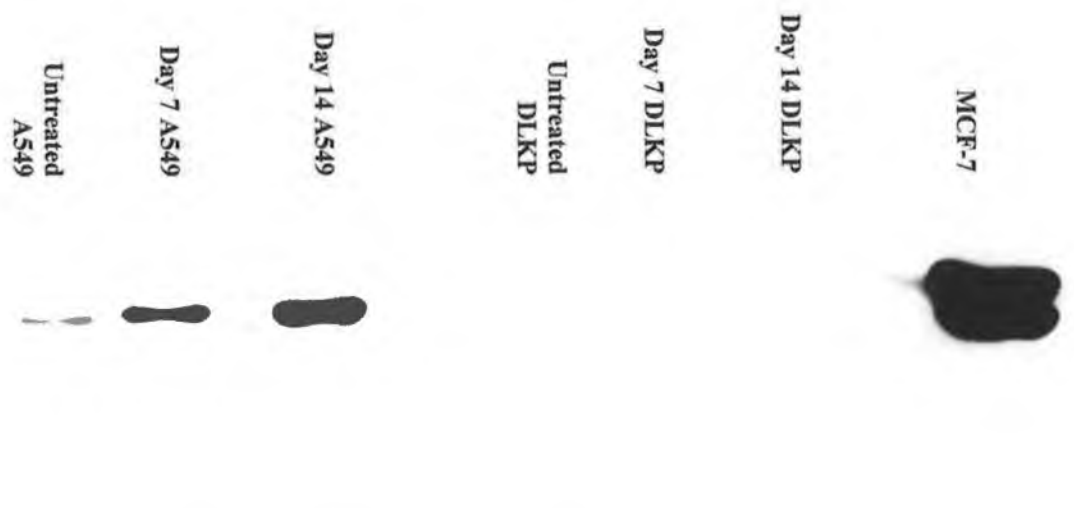


Figure 3.3.13 Western blot analysis for CK 19 expression in A549 and DLKP grown in HSM

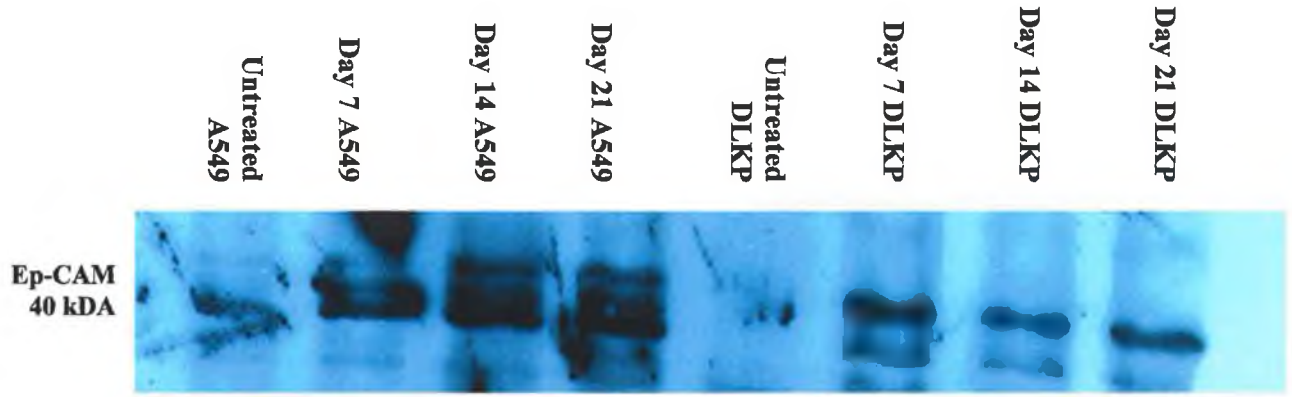


Figure 3.3.14 Western blot analysis of Ep-CAM expression grown in HSM

3.3.4 RT-PCR ANALYSIS OF MARKER PROTEIN mRNA EXPRESSION IN DLKP AND A549 FOLLOWING CULTURE IN HSM.

RT-PCR analysis was conducted to determine the effect of growth in HSM had on CK-8, CK-18, and CK-19 mRNA transcript levels.

- There was no CK-8 (figure 3.3.15), CK-18 (figure 3.3.16), CK-19 (figure3.3.17) mRNA detected in DLKP. These samples were in storage (at -80°C) for some time. Thus degradation of sample was deemed the most likely cause for this result.

- Growth in HSM by A549 did not appear to alter CK-8 mRNA expression levels (figure 3.3.15). In contrast a slight decrease is observed in CK-18 mRNA levels (figure 3.3.16), even though induction of protein was obtained by western blot analysis. Concurrently, a slight increase in CK-19 mRNA transcript levels was observed over the 21 days of growth (figure 3.3.17).

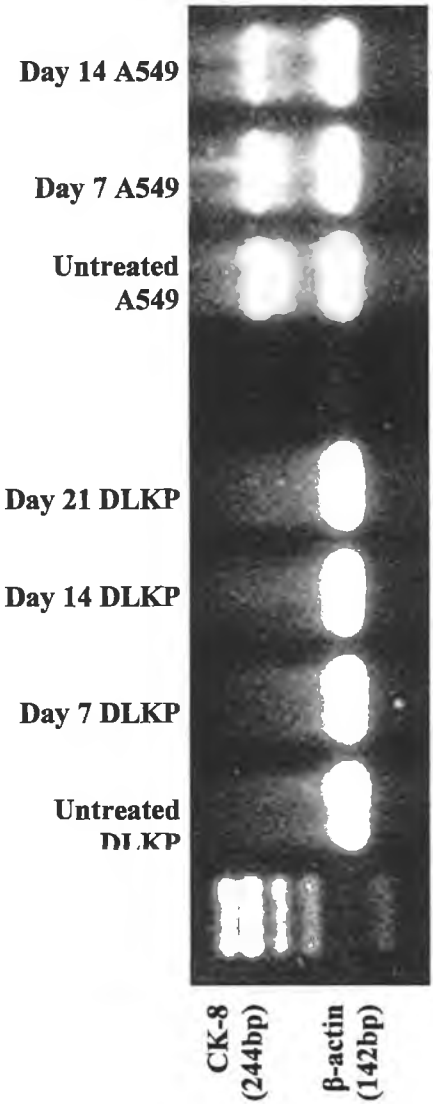

Day 14 A549
Day 7 A549
Untreated A549
Day 21 DLKP
Day 14 DLKP
Day 7 DLKP
Untreated DLKP
CK-8 (244bp)
β-actin (142bp)

Figure 3.3.15 RT-PCR analysis for CK-8 expression in A549 and DLKP grown in HSM.

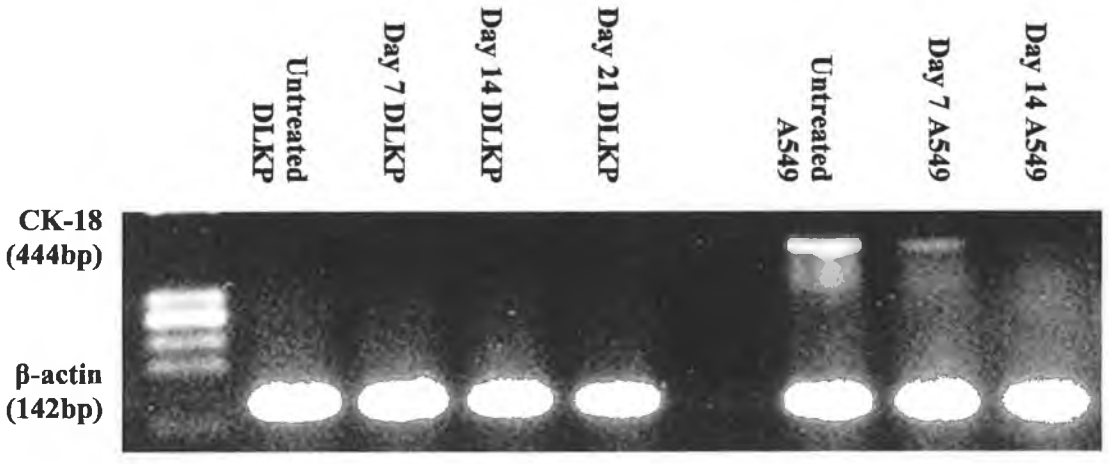


Figure 3.3.16 RT-PCR analysis for CK-18 expression in A549 and DLKP grown in HSM.

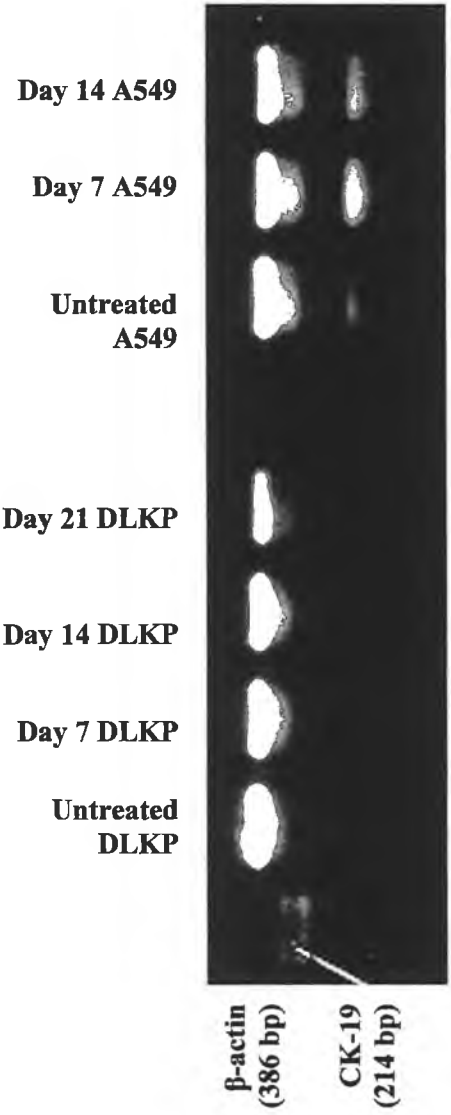

Day 14 A549
Day 7 A549
Untreated A549
Day 21 DLKP
Day 14 DLKP
Day 7 DLKP
Untreated DLKP
β-actin (386 bp)
CK-19 (214 bp)

Figure 3.3.17 RT-PCR analysis for CK-19 expression in A549 and DLKP grown in HSM.

3.3.5 PRELIMINARY INVESTIGATION ON THE EFFECTS OF DELETION OF SPECIFIC COMPONENTS IN HSM.

In order to assess what influence that various components of HSM had on the differentiation, experiments were conducted in which a specific factor was deleted from the medium. A549 cells were cultivated for 14 days and subsequently analysed for changes in morphology and selected marker proteins (cytokeratin-8, cytokeratin-18, cytokeratin-19, and Ep-CAM) summarised in table 3.3.1.

			HSM with various components deleted				
Marker	**Untreated.**	**Complete HSM.**	**EGF -Ve HSM.**	**Hydrocortisone - Ve HSM.**	**Oestrogen -Ve HSM.**	**Insulin -Ve HSM.**	**Cholera Toxin - Ve HSM.**
Cytokeratin-8	Positive	Same as Untreated	Same as Untreated Definitive assessment not possible	Same as Untreated Definitive assessment not possible	Same as Untreated Definitive assessment not possible	Same as Untreated Definitive assessment not possible	Same as Untreated Definitive assessment not possible
Cytokeratin-18	Positive	Same as Untreated	Same as Untreated Definitive assessment not possible	Same as Untreated Definitive assessment not possible	Same as Untreated Definitive assessment not possible	Same as Untreated Definitive assessment not possible	Same as Untreated Definitive assessment not possible
Cytokeratin-19	Positive	Induced expression	Induced expression	Strong induction of expression	Induced expression	No induction of expression	Very strong induction of expression
Ep-CAM	Positive	Induced expression	Induced expression	Strong induction of expression	Strong induction of expression	No induction of expression	Strong induction of expression

Table 3.3.1 Comparison of changes in A549 of marker proteins grown in various forms of HSM.

3.3.5.1 Alterations in A549 morphology and protein expression following deletion of EGF from HSM.

- Growth in HSM from which EGF had been deleted was accompanied by an alteration in morphology, when compared to untreated A549 cells and cells grown in complete HSM (figure 3.3.18 B). The cells still grew in tight colonies when compared, though some cells within these colonies showed increased flattening. This increased flattening was often accompanied by the formation of vacuoles within the cytoplasm.
- The expression of CK-8 in cells grown in HSM lacking EGF did not appear to alter (figure 3.3.19). However, assessment of this result was hampered by a saturation of signal, which means that subtle differences in protein level were not detectable.
- Similarly CK-18 expression did not appear to be altered following growth in HSM with EGF deleted. Once more assessment was hampered by a high signal strength (figure 3.3.20).
- Omission of EGF from HSM added did not seem to alter the ability of HSM to induce CK-19 expression in A549 cells (figure 3.3.21)
- Likewise the induction of Ep-CAM by HSM did not appear to be affected by the removal of EGF (figure 3.3.22).

3.3.5.2 Alterations in A549 morphology and protein expression following deletion of Hydrocortisone from HSM.

- Growth in HSM which had hydrocortisone deleted, had a very altered morphology and was unlike either untreated A549 or cells grown in complete HSM. The cells appeared to be much smaller in cell volume with a 'spiky' appearance and grew in loose colonies (figure 3.3.18 C). The culture also contained a sub-population of cells, which possessed a flattened morphology and usually grew as isolated cells.
- HSM from which hydrocortisone had been withdrawn did not appear to alter the expression of CK-8 (figure 3.3.19), though due to the high signal strength assessment was difficult.

- Likewise the expression of CK-18 did not appear to be altered in HSM from which hydrocortisone was deleted (figure 3.3.20). However, in common with the CK-8 result, assessment was difficult due to the strong signal strength.
- HSM from which hydrocortisone was deleted, induced strong expression of CK-19 (figure 3.3.21). The strength of this induction was greater than the induction of CK-19 expression induced by complete HSM.
- Similarly the expression of Ep-CAM was greatly increased in HSM lacking hydrocortisone (figure 3.3.22). The level of induction was greater than the induction observed in complete HSM.

3.3.5.3 Alterations in A549 morphology and protein expression following deletion of oestrogen from HSM.

- The growth of A549 in oestrogen deleted HSM did not appear to alter the cellular morphology greatly, with the cells growing in tight colonies (figure 3.3.18 D).
- HSM lacking oestrogen did not appear to alter the expression of CK-8 (figure 3.3.19). Assessment of this result was hampered by a saturation of signal, which prevented subtle differences in protein level to be observed.
- Similarly, CK-18 expression did not appear to be altered following growth in HSM with oestrogen deleted. Thought assessment was once more hindered by a high signal strength (figure 3.3.20).
- The removal of oestrogen from HSM did not obviously alter the ability to induce the expression of CK-19 (figure 3.3.21).
- HSM which was lacks oestrogen appeared to have an enhanced ability to induce Ep-CAM expression (figure 3.3.22)

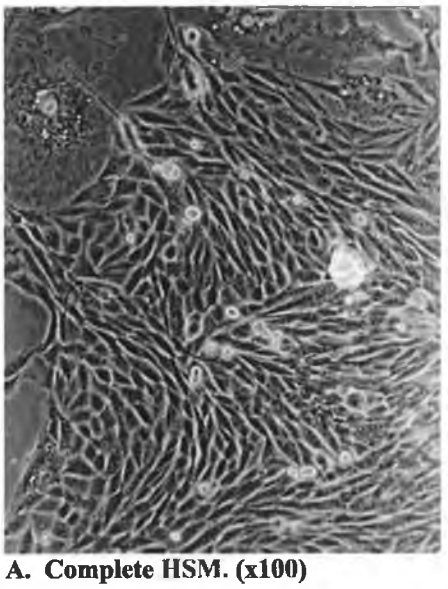
3.3.5.4 Alterations in A549 morphology and protein expression following deletion of insulin from HSM.

- The withdrawal of insulin from HSM did not appear to alter the cellular morphology of A549 greatly from that of untreated A549 cells (figure 3.3.18 E)

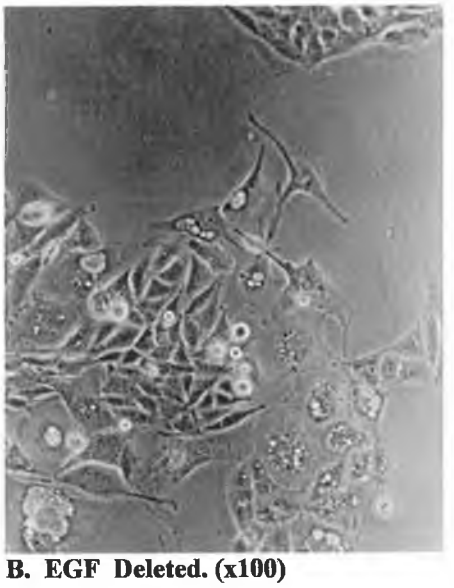
- HSM lacking insulin did not appear to alter the expression of CK-8, though high signal level made it difficult to be conclusive (figure 3.3.19).
- The expression of CK-18 did not appear to be induced in cells grown in insulin deleted HSM. This result cannot be taken as being completely definite due to the high signal level (figure 3.3.20).
- The removal of insulin did not appear to alter the ability of HSM to induce CK-19 expression (figure 3.3.21).
- Insulin depleted HSM appeared to possess a decreased ability to induce Ep-CAM expression (figure3.3.22).

3.3.5.5 Alterations in A549 morphology and protein expression following deletion of cholera toxin from HSM.

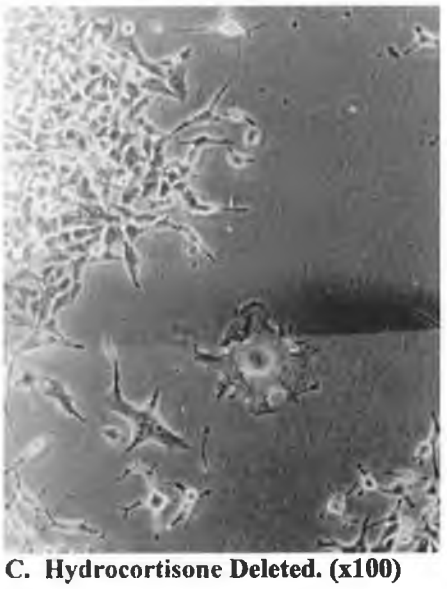
- The deletion of cholera toxin from HSM induced a more squamous morphology, with an apparent increase in the number of cytoplasmic granules (figure 3.3.18 F).
- HSM from which cholera toxin had been withdrawn did not appear to alter the expression of CK-8 (figure 3.3.19). Though due to the high signal strength it is difficult to make a conclusive judgement.
- Likewise the expression of CK-18 did not appear to be altered in HSM from which cholera toxin was deleted (figure 3.3.20). However in common with the CK-8 result, assessment was difficult due to the strength of the signal.
- The removal of cholera toxin from HSM, increased the ability of HSM to induce CK-19 expression (figure 3.3.21). The strength of this signal was substantially greater then that observed from complete HSM.
- HSM from which cholera toxin was deleted induced a much stronger expression of Ep-CAM than that observered with cells grown in complete HSM (figure 3.3.22).



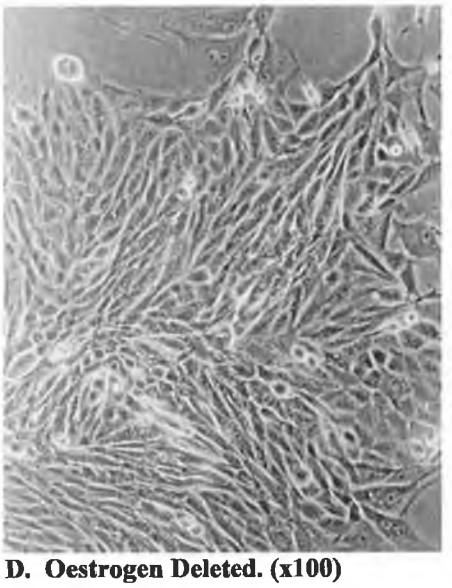
A. Complete HSM. (x100)



B. EGF Deleted. (x100)



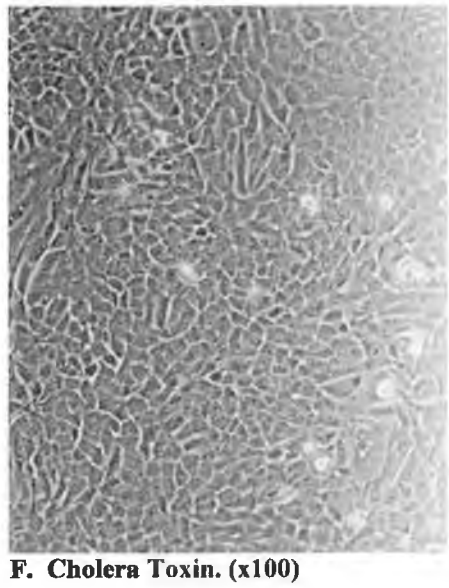
C. Hydrocortisone Deleted. (x100)



D. Oestrogen Deleted. (x100)

Figure 3.3.18 Morphology of A549 grown in HSM with various components deleted.

E. Insulin Deleted. (x100)



F. Cholera Toxin. (x100)

Figure 3.3.18 (Continued) Morphology of A549 grown in HSM with various components deleted.

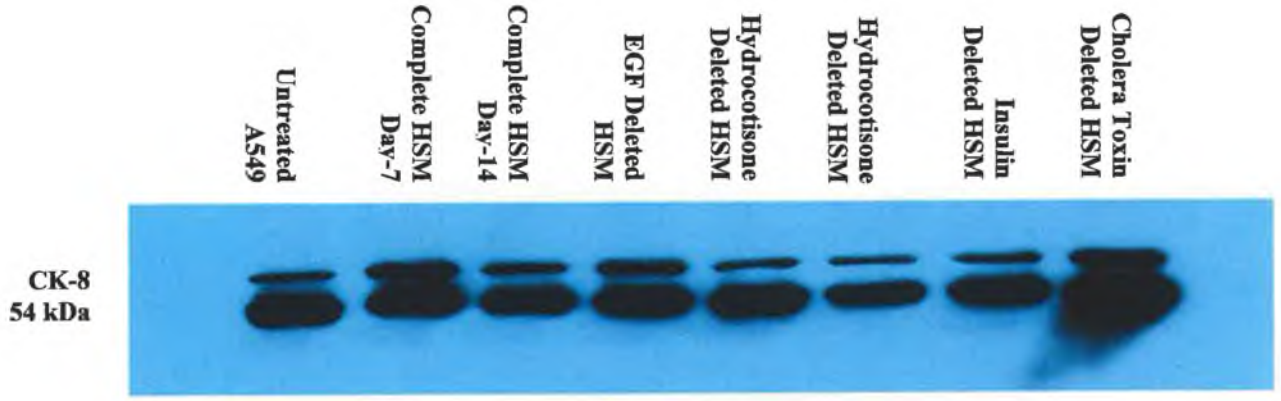


Figure 3.3.19 Western blot analysis of cytokeratin-8 (CK-8) expression in A549 cells following growth in HSM with various components deleted.

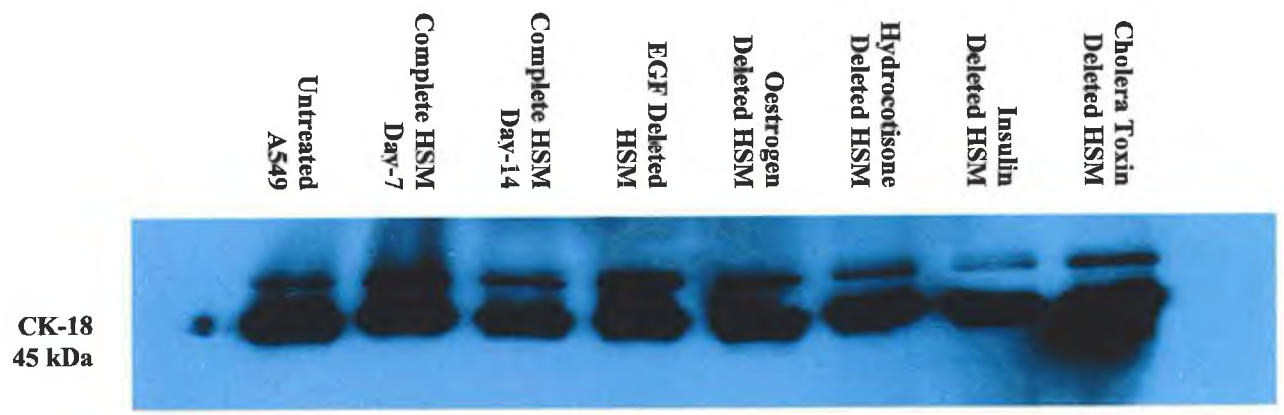


Figure 3.3.20 Western blot analysis of cytokeratin-18 (CK-18) expression in A549 cells following growth in HSM with various components deleted

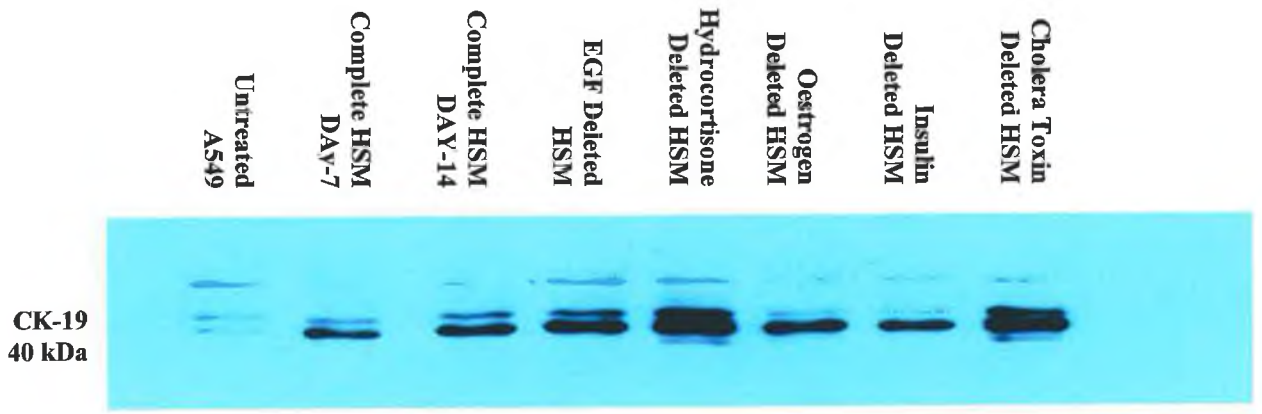


Figure 3.3.21 Western blot analysis of cytokeratin-19 (CK-19) expression in A549 cells following growth in HSM with various components deleted.

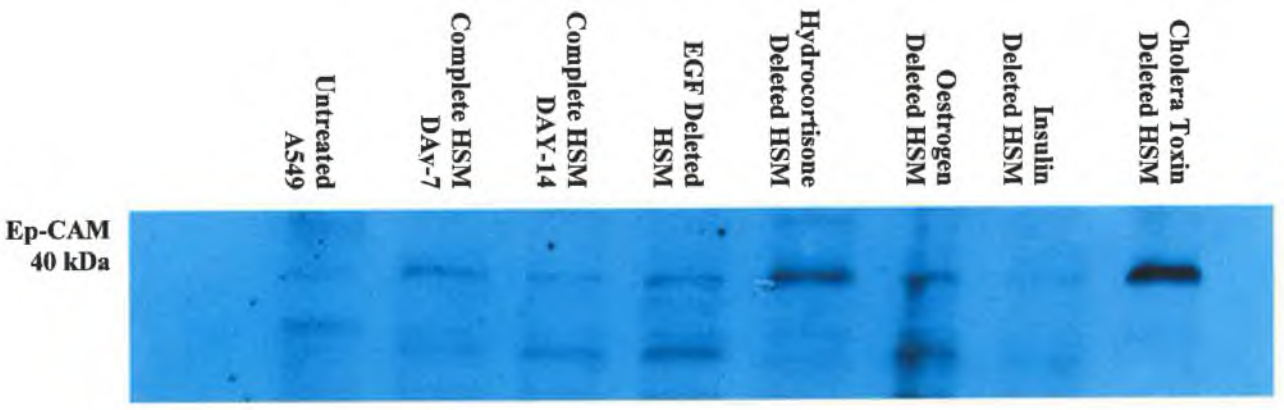


Figure 3.3.22 Western blot analysis of Ep-CAM expression in A549 cells following growth in HSM with various components deleted.

3.4 DEVELOPMENT OF *IN VITRO* MODELS OF DIFFERENTIATION WITH PRIMARY CULTURES OF NORMAL LUNG AND LUNG CARCINOMA CELLS.

3.4.1 PRIMARY CUTURE AND PRELIMINARY DIFFERENTIATION STUDIES OF TYPE II PNEUMOCYTES.

Type II pneumocytes were isolated from rat lung. These, were used to investigate the behaviour of normal cell populations *in vitro* and to establish a foundation for their use in modelling differentiation

3.4.1.1 Time-Lapse observations of isolated rat type II pneumocytes.

To investigate the effect of seeding density on proliferation and differentiation in isolated rat type II pneumocytes cells time-lapse studies were performed. Freshly isolated rat type II pneumocyte cells were plated at low density 5 x 10^5 and at high density 1.5 x 10^6 per well of a 12 well plate. These were then allowed to attach for 16 hours, before the media was changed and the culture observed by time lapse.

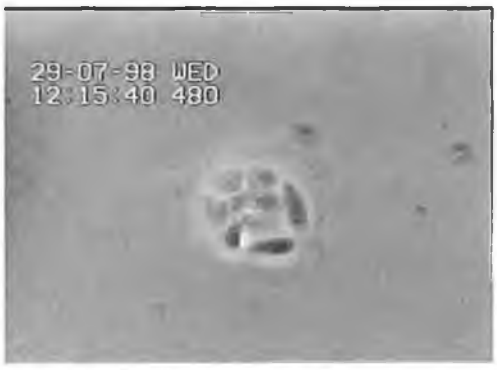
In summary both low- and high-density cultures exhibited no proliferation from 16 hours after isolation till the end of the experiment, which occurred when the cells died by apoptosis. During the assay the cells in both low- and high-density cultures became progressively flatter and larger, with a small decrease in lamellar body content. This is suggestive of the type II pneumocytes adopting a type I cell morphology. The cells in the high-density culture appeared to change their morphology and enter apoptosis slightly later then those type II cells in low-density culture.

Figure 3.4.1 shows photos of selected time points from the time-lapse video of a low-density culture (LD). The observations made for this low-density culture of type II pneumocyte are summarised in table 3.4.1.

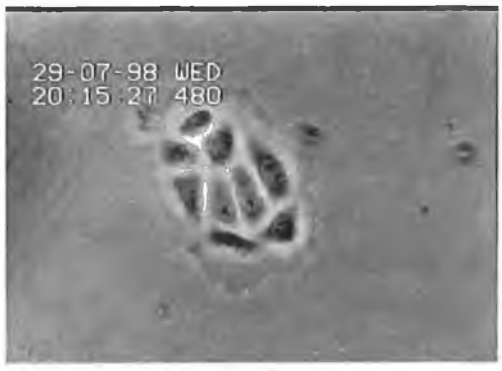
Figure 3.4.2 shows photos of selected time points from the time-lapse video of a high-density culture (HD). The observations of these cultures are summarised in table 3.4.2

Time : 16 Hrs. Fig. 3.4.1 A	9 cells are present in a colony. The lamellar bodies are not very clear.
Time : 24Hrs. Fig. 3.4.1 B	A large increase in the size of the colony has occurred, but with not increase in cell number.
Time : 32 Hrs. Fig. 3.4.1 C	The cells in the colony are still spreading out. The colony still contains 9 cells
Time : 24 Hrs. Fig. 3.4.1.D	The cells are now very flat and cover a large surface area.
Time : 48 Hrs. Fig. 3.4.1 E	Some cells have died by apoptosis
Time : 72 Hrs. Fig. 3.4.1 F	All cells have died buy apoptosis.

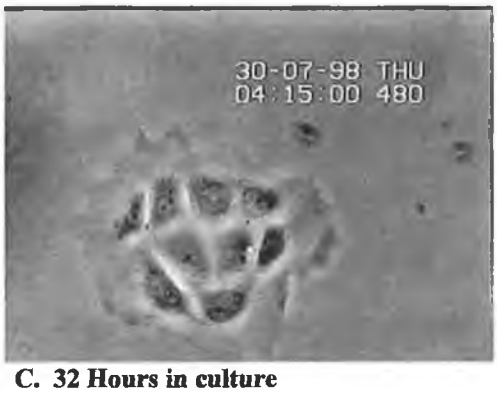
Table 3.4.1 Summary of time-lapse observations for low density culture of rat type II pneumocytes (figure 3.4.1)



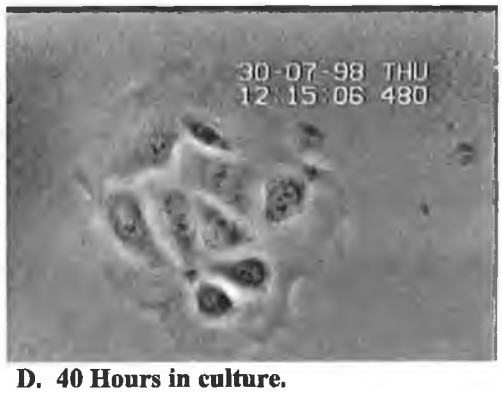
A. 16 Hours in culture.



B 24 Hours in culture.

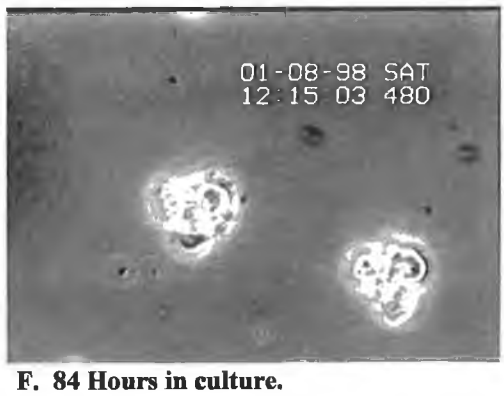


C. 32 Hours in culture



D. 40 Hours in culture.

E. 50 Hours in culture

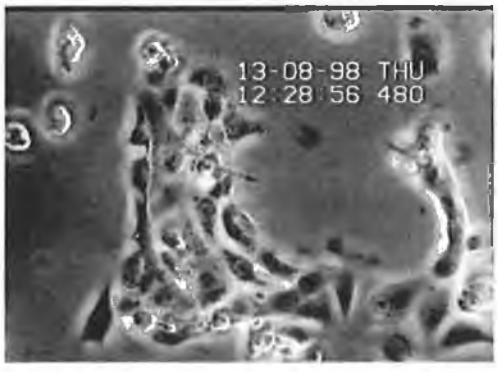


F. 84 Hours in culture.

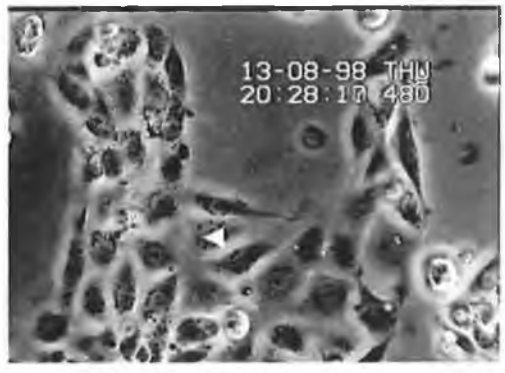
Figure 3.4.1 Rat type II pneumocytes cultured at low density followed by time-lapse video microscopy (x400).

Time : 16 Hrs. **Fig. 3.4.2 A**	A large colony of cells is present (approx. 48 cells). These cells express clear lamellar bodies (arrow).
Time : 24 Hrs. **Fig. 3.4.2 B**	The cells are now covering a larger surface area. However there has been no cell division observed and the cell number is still approx. 48
Time : 32 Hrs. **Fig. 3.4.2 C**	The cells now cover a large surface area. The cells while obviously larger and flatter still possess lamellar bodies.
Time : 40 Hrs. **Fig. 3.4.2 D**	The cells now cover all the surface area. This is due to the cells spreading rather then cell proliferation. The cell number is still approx. 48.
Time: 48 Hrs. **Fig. 3.4.2 E**	Cells appear to have flattened further and some cells have begun to die by apoptosis
Time : 72 Hrs. **Fig. 3.4.2 F**	More cells have died by apoptosis. No cell proliferation has occurred.

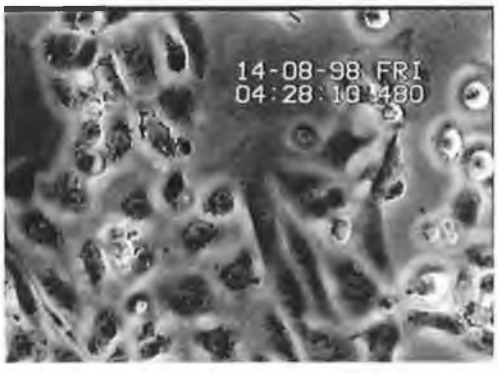
Table 3.4.2 Summary of time lapse observations for high density culture of rat type II pneumocytes. (figure 3.4.2)



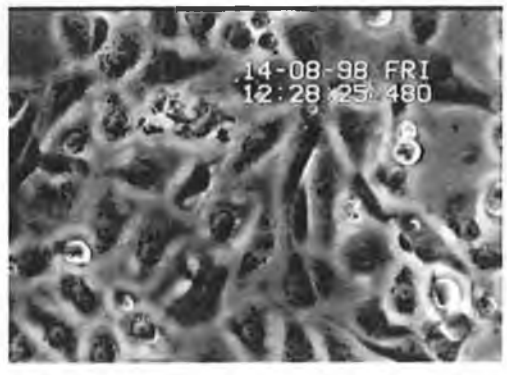
A. 16 Hours in culture



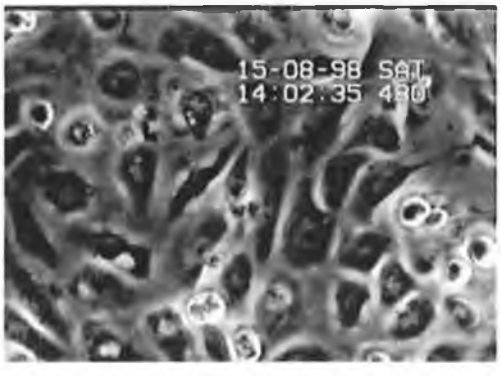
B. 24 Hours in culture.



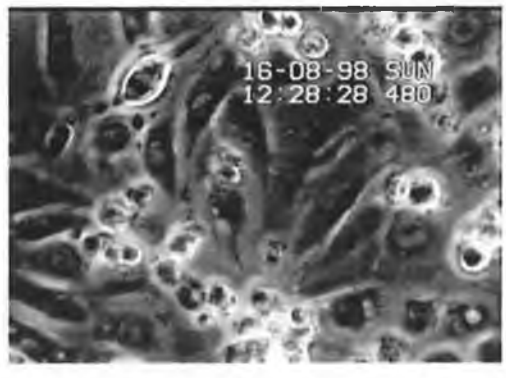
C. 32 Hours in culture.



D. 40 Hours in culture.



E. 50 hours in culture.



F. 84 Hours in culture.

Figure 3.4.2 Rat type II pneumocytes cultured at high density followed by time-lapse video microscopy (x400).

3.4.1.2 Immunocytochemistry of isolated rat type II pneumocytes.

Preliminary investigations of changes in antigenic expression in rat type II pneumocytes over time in culture, were conducted. The type II pneumocytes were cultured for 8 days and were stained at fixed time points. The antigen chosen for study were:

- Alkaline phosphatase, which is an enzyme important in the lipid surfactant pathway, thus it is indicative of type II pneumocytes.
- Surfactant protein D, a kind gift from Dr Henk Haagsman (University of Utrecht), it is one of the component proteins of the surfactant produced by type II pneumocytes.
- Cytokeratin 18, is one of the main structural proteins of type I pneumocytes.
- Cytokeratin 19, is one of the main structural proteins of type II pneumocytes.

A summary of the results obtained is presented in table 3.4.3.

	Day 0	Day 2	Day 4	Day 6	Day 8
Alkaline Phosphatase	90 – 95%	55 – 60%	50 - 45 %	25 - 30 %	15 - 10 %
Surfactant Protein D	95 -100 %	70 - 75 %	35 - 30 %	10 - 15 %	5 - 0 %
Cytokeratin-18	5 - 10 %	5 - 10 %	10 - 15 %	20 - 25 %	40 - 45 %
Cytokeratin-19	80 - 85 %	55 - 60 %	10 - 15 %	10 - 15%	5 - 10 %

Table 3.4.3 Percentage of cells stained for various markers over time in culture.

3.4.2 SELECTION AND CHARATERISATION OF HUMAN LUNG CARCINOMA SAMPLES.

The aim of this work was to establish the foundation for the routine primary culture of human carcinoma cells. Such cultures would form the bases of an *in vitro* model for studying differentiation in human lung carcinomas.

A total of 52 lung carcinoma samples were utilised in the investigation. A number of oesophageal tumour samples were also obtained.

The majority of samples came from patients undergoing lobectomy (removal of one or more constituent lobes of the lung or pneumonectomy (removal of one of the lungs). This type of resection for large tumours was normally reserved for non-small cell lung carcinoma. The second source of tissue came from investigative procedures such as bronchoscopy and mediastinoscopy. Both procedures are usually performed sequentially on the patient. The amount of tissue available from these procedures is very limited, usually in the range of 2-3 cm^3. The tissue samples were placed immediately into cool (4 ^{o}C) transport media upon resection and transported at 4 ^{o}C promptly to the lab.

At the time of collecting the samples the histology may not have been known or available. However, most samples appear to have been from patients with either adenocarinoma or squamous cell carcinoma. It was not always clear if the samples were from patients with malignant or benign tumours. In a limited number of cases some tissue specimens were metastases from other tissues e.g. an ovarian tumour, a sarcoma of the uterus, and melanoma. Within the 52 samples a great deal of tissue heterogeneity existed e.g. size, texture, associated adipose tissue, necrosis and vascularisation.

3.4.3 ENZYMATIC DISAGGREGATION OF TISSUE SAMPLES.

Two types of enzymatic digestion were investigated for their ability to dissagregate sample tissue and generate cultures of viable epithelial cells.

- The first method utilised trypsin 2 ml of 10x Gibco BRL trypsin in 18ml of MEM supplemented with 10U/ml of DNase I. In which the tissue was digested for 2 x 30 minutes.
- The second treatment utilised 0.4 mg/ml Collagenase A, 0.6 mg/ml Dispase, 0.6 mg/ml Pronase E in MEM supplemented with 10 U/ml of DNase I. the tissue was digested for 2 x 30 minutes.

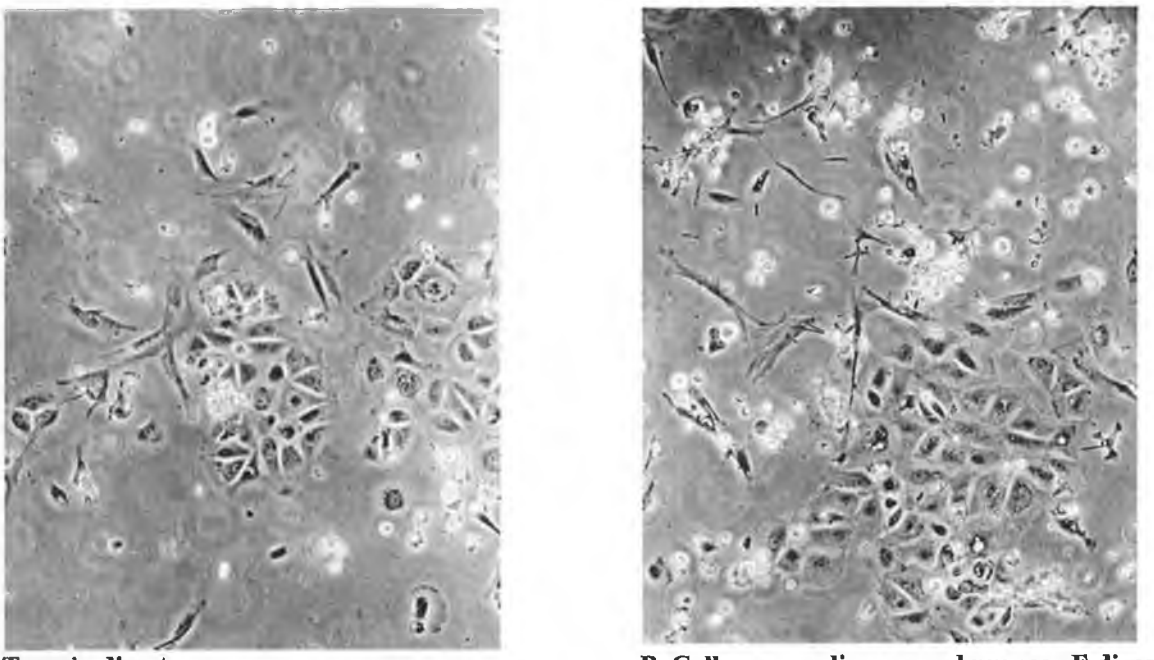
See methods section 2.15 for a more detail account of the isolation procedure.

Neither method of enzymatic treatment appeared to confer an advantage for the isolation and culture of epithelial cells, (figure 3.4.3 A and B).
Epithelial colonies were usually obtained from the enzymatic digestion of the sample tissue. These usually only lasted for 1 or 2 passages at most and never reached confluency. The cultures usually became dominated by fibroblasts (figure 3.4.4).

Explants were also investigated for their ability to promote epithelial growth *in vivo*. These took longer for cells to become established and appeared to suffer similar amounts of fibroblast overgrowth as those cultures generated by enzymatic methods (figure 3.4.5).

A number of methods were investigated for their ability to prevent fibroblast overgrowth. These included using D-valine MEM as the basal media, the supplementation of the culture media with putrescence, differential trypsinisation, and panning of cells during isolation. None of these methods conveyed an advantage over a long culture time. The panning for fibroblasts during isolation was incorporated into the final protocol adopted as it may allow the epithelial cells some selective advantage during the initial culture period.

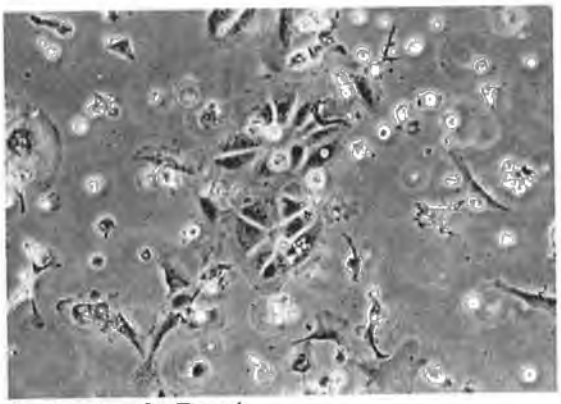
The ability to obtain relatively pure cultures of lung carcinoma epithelial cells and to work with such cultures beyond 1 passage was very poor. The one culture most successful was from a highly malignant melanoma of the oesophagus, this culture was known as WS-111 (figure 3.4.6 A and B). The culture was passaged 3 times over two months before the melanoma cells died. The culture was slow in its growth, and contained a handful of contaminating fibroblast like cells, these cells subsequently took over (figure 3.4.6 C and D).



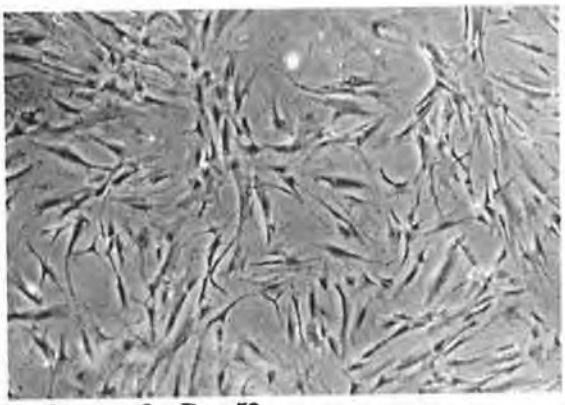
A. Trypsin digest.

B. Collagenase, dispase, and pronase-E digest

Figure 3.4.3 Comparison of primary culture MS-25.6 generated using different enzyme systems (x100).



A. Passage 0 - Day 4.



B. Passage 3 – Day 52.

Figure 3.4.4 Overgrowth of primary culture GOK-65 with fibroblast (x100).

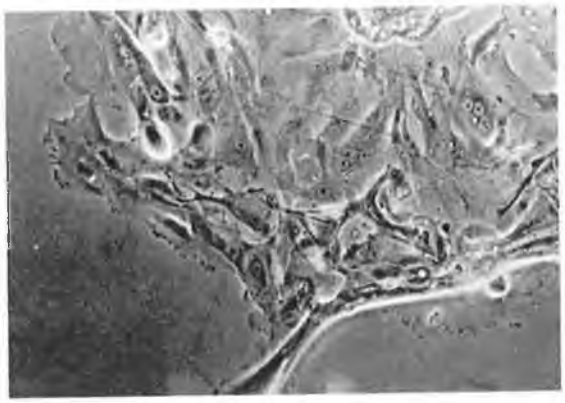
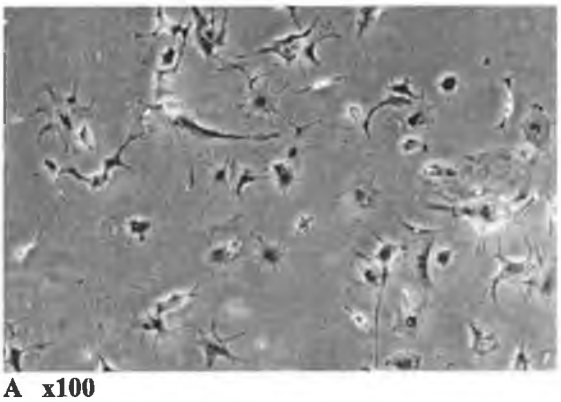
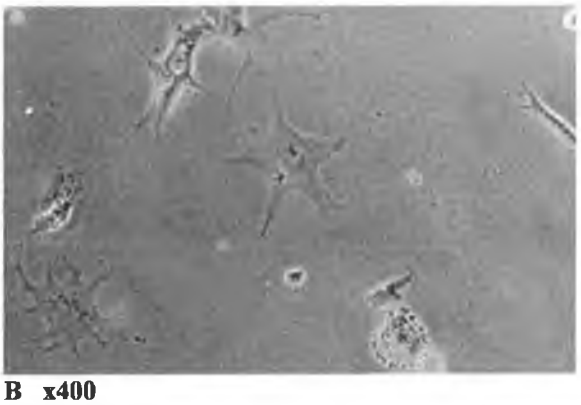


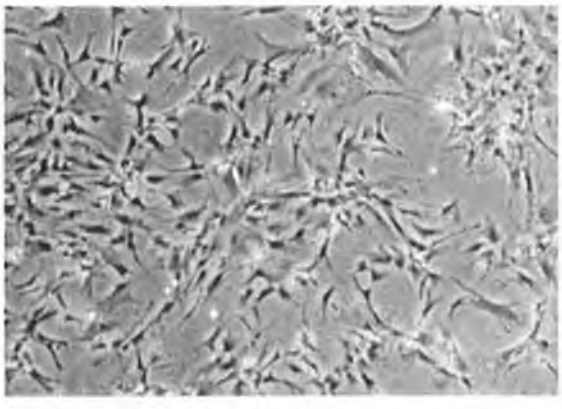
Figure 3.4.5 Outgrowth from explant after 18 days (x100).



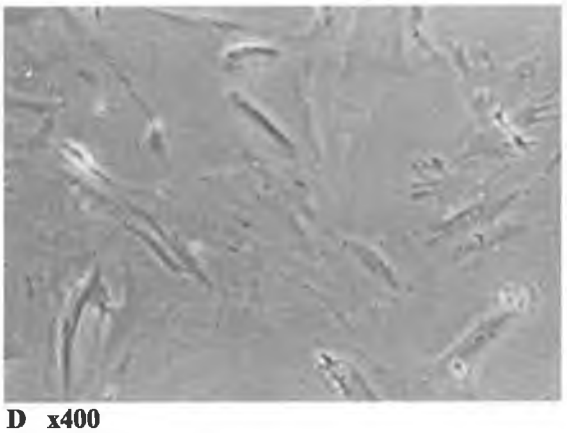
A x100



B x400



C x100



D x400

Figure 3.4.6 Primary culture WS-111 at passage 0 (A) and (B) showing cells with a myeloma morphology; at passage 6 (C) and (D) showing overgrowth and replacement with fibroblast cells.

3.4.4 INVESTIGATION INTO CYTOSKELETAL EXPRESSION IN FIBROBLAST CELLS *IN VITRO*.

This study arose serendipitously when it was decided to use fibroblast cells derived from primary culture as negative controls for cytokeratin expression. Surprisingly, these cells proved to be positive.

Subsequent immunocytochemistry studies were performed on three primary cultures of fibroblasts derived from lung carcinoma samples, which were designated -

JK-13.10

HG-18.10

BD-25.10

Three normal fibroblast cell cultures available from external cell culture collections were also analyzed

AG02603 Normal lung clinically unaffected (Source: NIH; Institute of aging)

AG02602A Normal skin clinically unaffected (Source : NIH; Institute of aging)

CCD-37lu Normal lung (Source: ATCC)

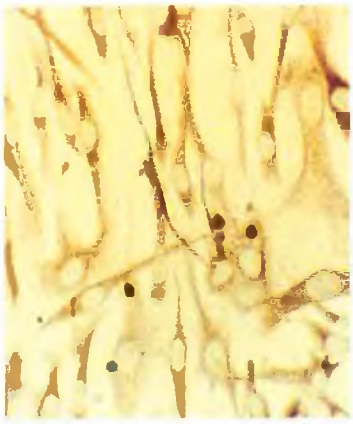
The normal fibroblast cells AG02603 (lung) and AG02602A (skin) are a matched pair from the same patient. The skin cells are supplied at a later passage.

Immunocytochemistry was carried out using antibodies to the following antigens; Pan-Cytokeratin; Cytokeratin 8; Cytokeratin 18 and the results obtained are summarised in table 3.4.4.

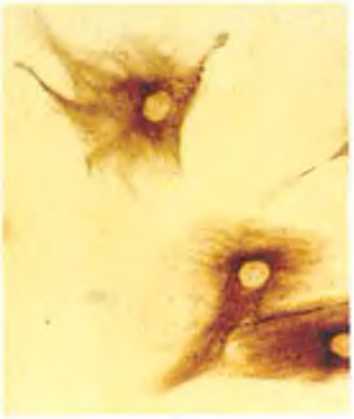
Though all fibroblast cells stained positive for pan-cytokeratin (figure 3.4.8) the staining for specific cytokeratins was variable (figures 3.4.9 and 3.4.10). For example the JK-13.10 fibroblasts were negative for cytokeratins 8 and 18, the HG-18.10 fibroblast cells were weakly positive for cytokeratin 8 and positive for cytokeratin 18, and BD-25.10 fibroblasts strongly positive for cytokeratins 8 and 18 (figures 3.4.9 and 3.4.10).

	Pan-Cytokeratin	Cytokeratin-8	Cytokeratin-18
AG02603 (Normal lung)	Positive	Positive	Positive
AG02602A (Normal skin)	Positive	Positive	Positive
CCD-34lu (Normal lung)	Positive	Positive	Positive
JK-13.10	Positive	Negative	Negative
HG-18.10	Positive	Weakly-Positive	Positive
BD-25.10	Positive	Positive	N/A

Table 3.4.4 Summary of immunocytochemical analysis for cytokeratin expression in fibroblasts.

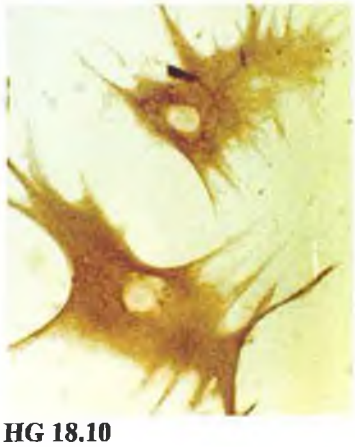


CCD 37 lu

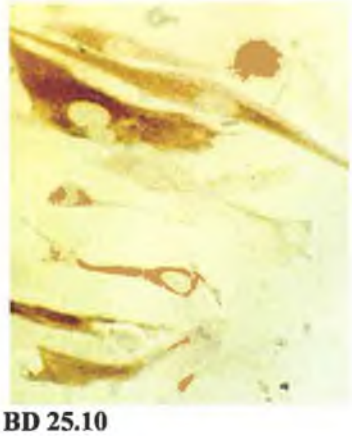


AG 02602A Skin

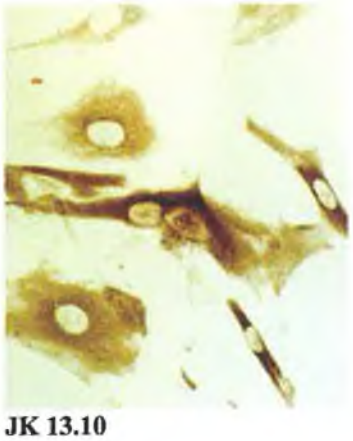
AG02603 Lung



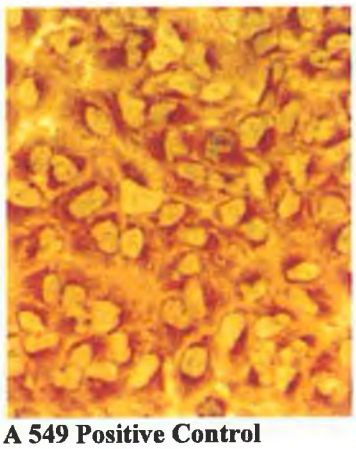
HG 18.10



BD 25.10

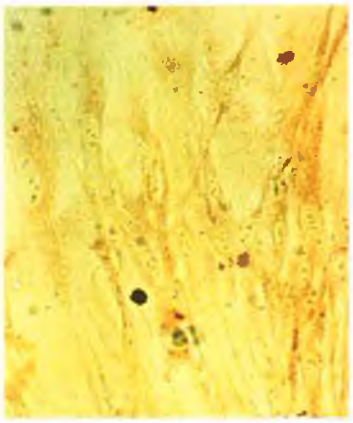


JK 13.10

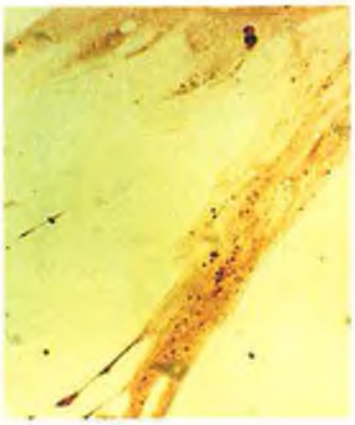


A 549 Positive Control

Figure 3.4.8 Staining for pan-cytokeratin in various fibroblast cultures (x400).

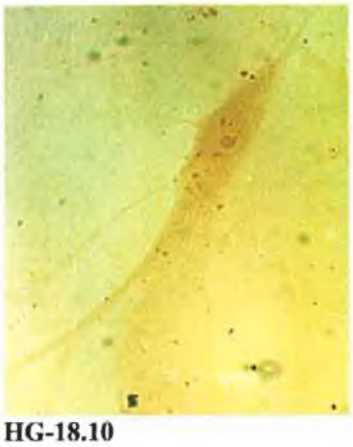


CCD 37 Lu



AG02602A (Skin)

AG02603 (Lung)



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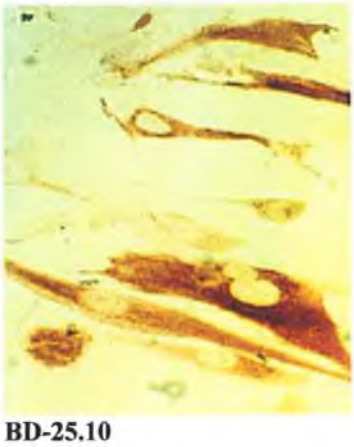

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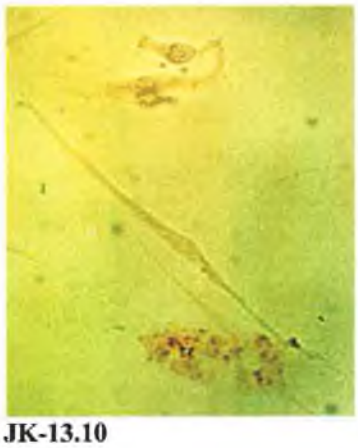
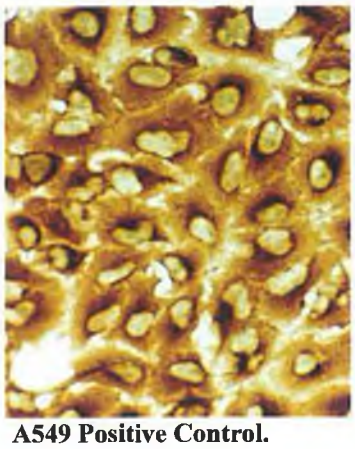


JK-13.10

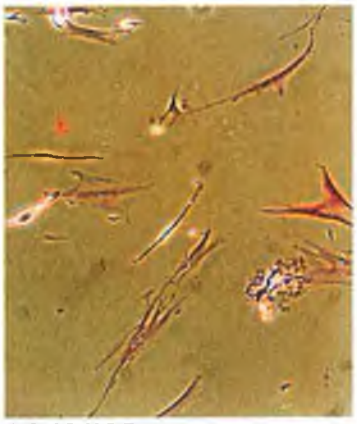


A549 Positive Control.

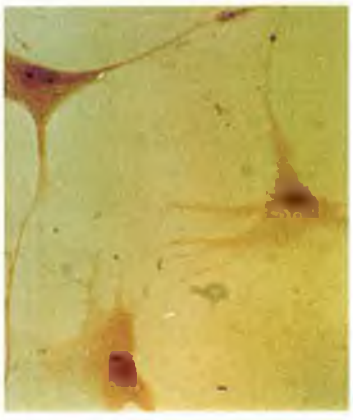
Figure 3.4.9 Staining for cytokeratin-8 in various fibroblast cultures (x400).

CCD-37lu

AG 02603 Skin

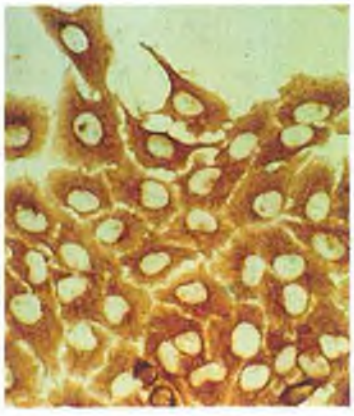


AG 02603 Lung



HG-18.10

JK-13.10



A549 Positive control

Figure 3.4.10 Staining for cytokeratin 18 in various fibroblast cultures (x400).

4.0 DISSCUSSION.

4.1 GENERAL INTRODUCTION.

The lung is a complex organ consisting of over 40 different cell types (Plopper, 1996), whose development has been divided into four chronological stages; I) the pseudoglandular stage, II) the canalicular stage, III) the terminal sac stage, and IV) the alveolar stage. It is only recently from studies of *Drosophila* mutants that tentative identification has been made of some of the developmental genes involved in lung morphology e.g. *trachealess* (Wilk *et al.*, 1996) and *branchless* (Glazer and Shilo, 1991). While progress has been made in the developmental genetics of lung morphogenesis, the differentiation pathway and the genes involved in development of specific cell types in the lung are still largely unknown. Research in this area has been hampered by the lack of identification of a stem cell population *in vivo* and/or the lack of *in vitro* cell line models to study lung cell differentiation.

What is known about lung cell differentiation comes from studies of the terminal parts of the differentiation tree where a specific basal cell will give rise to a discrete differentiated cell type, e.g. type II pneumocytes give rise to type I pneumocytes (Adamson and Bowden, 1979). Steps in the differentiation pathway earlier than this are unknown. It is hypothesised by Emura (1997) that a hierarchy exists within the lung cell differentiation pathway. His model envisages that a single stem cell exists for both the pseudostratified and simple epithelia. This single stem cell population conducts its role through various predifferentiated secretory cell populations, which in turn give rise to various functional cell types that may differentiate into further terminal stage cells (figure 1.3.3)

One of the principal aims of this thesis was to investigate the possible role of the cell-cell adhesion molecule Ep-CAM in epithelial lung cell line differentiation, following BrdU treatment. The study utilised the poorly differentiated lung carcinoma cell line DLKP and the lung adenocarcinoma cell line A549 as models. The ability of halogenated thymidine analogues to induce differentiation was investigated. The investigation utilised Ep-CAM as a marker for differentiation to complement on going

investigations of changes recently discovered in this laboratory in cytokeratin expression and β_1 integrin expression (McBride *et al.*, 1999; Meleady and Clynes, manuscript submitted).

4.2 INDUCTION BY BrdU OF Ep-CAM EXPRESSION IN LUNG CELL LINES.

It is speculated that the cell line DLKP, which was derived from a lung tumour which was diagnosed as a poorly differentiated lung carcinoma, may serve as a model for a stem cell like-population of the lung (McBride *et al.*, 1998). It thus may represent a valuable model for investigating the role of Ep-CAM in differentiating lung epithelial cells. The studies included a comparison with the cell line A549, which represents a more differentiated lung cell type, as it has been reported to express features of a type II pneumocyte cell (Lieber *et al.*, 1976).

4.2.1 BrdU INDUCES Ep-CAM EXPRESSION IN BOTH DLKP AND A549.

Ep-CAM is described as having a wide distribution in normal human epithelia with expression found on the most simple, columnar and pseudostratified epithelia including bronchiolar and alveolar cells (Mombuerg *et al.*, 1987)

Analysis of Ep-CAM expression by immunocytochemistry with the anti- Ep-CAM antibodies VU-1D9 and 323/A3 showed DLKP to be negative for Ep-CAM (figure 3.1.1) whereas A549 to be slightly positive for Ep-CAM (figure 3.1.2).

The lack of Ep-CAM expression in DLKP and the low level of expression in A549 could be considered unusual, as Ep-CAM expression is reported to increase in most adenocarcinomas and *de novo* expression frequently occurs in carcinomas from squamous epithelia (Litvinov *et al.*, 1996; Varki *et al.*, 1984). In particular, strong Ep-CAM expression has been reported for small cell lung carcinoma cell lines (Moldenhauer *et al.*, 1987).

Following exposure to 10 μM BrdU for 7 days, the morphology of both A549 and DLKP changed with the cells becoming enlarged and flattened in appearance. Immunocytochemical analysis revealed that the changes observed in morphology were accompanied by changes in the expression of Ep-CAM protein. DLKP was shown to express Ep-CAM *de novo* following BrdU treatment for 7 days. This staining appeared strongest in the cell membrane indicating that the protein was likely to be functional and to be active in the cellular physiology of BrdU-treated DLKP cells. In A549 cells immunocytochemical analysis revealed a similar increase and staining pattern for Ep-CAM protein expression following exposure to 10μM BrdU exposure.

While immunocytochemistry has the advantage of detecting changes in sub-populations, which might not be noticeable in bulk methods such as western blotting, it should only be regarded a qualitative method, since changes in cell morphology may give the appearance of changes in protein expression. For this reason, western

blot analysis was performed (section 3.1.2) to obtain quantitative results. DLKP and A549 were treated for 21 days with 10 μM BrdU and time points were taken at day 7, day 14 and day 21. Treatment of DLKP with 10μM BrdU seemed to trigger *de novo* expression of Ep-CAM; as in untreated cells no protein could be detected but after 7 days of treatment a protein band was detectable (figure 3.1.3). The expression of protein continued to increase until day 14, at which point it appears to have plateaued with no visible increase occurring between day 14 and day 21 of BrdU treatment. In A549 a similar pattern was observed (figure 3.1.4); untreated cells were found to express a low level of Ep-CAM which increased within 7 days of BrdU treatment. The expression of Ep-CAM continued to increase until day 14 at which point a plateau level was also reached.

Litvinov *et al.* (1996) reported that Ep-CAM was expressed in a stem cell-like population of cervical epithelial cells and that this expression was decreased when these stem cells differentiated into squamous epithelia. In metastatic disease states of the uterine cervix the expression patterns of the simple epithelial cell cytokeratins 8 and 18 and of Ep-CAM were identically high (Litvinov *et al.*, 1996).

Previous studies in our laboratory have shown that DLKP lacks the major cytokeratins, including cytokeratins 8 and 18 (McBride *et al.*, 1999). The expression of cytokeratins 8 and 18 are indicative of early epithelial differentiation (Daly *et al.*, 1998). Thus the lack of these cytokeratins and of Ep-CAM expression would appear to confirm the theory that DLKP represents a cell population early in the differentiation pathway of lung epithelia.

It could be theorised that BrdU induces DLKP into a cell phenotype that represents an early stage in lung stem cell differentiation, involving changes in the cytoskeletal structure (induction of cytokeratin expression), a change in cell-cell binding as suggested by the induction of Ep-CAM, and a change in the recognition and affinity of extra-cellular matrix as implied by the induction of $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins (McBride *et al.*, 1999; Meleady and Clynes, manuscript submitted). It could be speculated that this stage of differentiation occurs prior to the expression of more

specialised lung cell features such as surfactant proteins, microvilli, etc., and may be within stage II of the scheme proposed by Emura (section 1.3.2).

4.2.2 Ep-CAM INDUCTION BY BrdU IS AT THE POST-TRANSCRIPTIONAL LEVEL.

To investigate what effect BrdU had on the mRNA transcript levels for Ep-CAM, RT-PCR analysis was performed. The transcript levels were assessed over 21 days of BrdU treatment with time points of 7, 14 and 21 days.

Little is known about the regulation of Ep-CAM expression. Northern blot analysis identifies a 1.5kb mRNA species, which contains some recognition sequences that occur in the post-transcriptional control of certain proto-oncogenes and inflammatory mediators (Perez and Walker, 1989). Included in the 3'-non coding region of the Ep-CAM mRNA transcript is the consensus sequence TTATTTAT, associated with the inflammatory mediators (Perez and Walker, 1989). In addition, the 3'-non coding region of Ep-CAM contains the sequence ATTTA, which is a proposed recognition signal for the degradation processing of mRNA coding for a number of proto-oncogenes, lymphokines and cytokines (Perez and Walker, 1989).

In both DLKP and A549, constitutive expression of Ep-CAM at the mRNA level occurred in untreated cells (figure 3.1.5). Following treatment with BrdU no change in levels of Ep-CAM mRNA was observed in either DLKP or A549. This result suggests that the BrdU induced *de novo* Ep-CAM protein expression in DLKP (and increased its expression in A549) is via post-transcriptional control mechanisms.

Studies in our laboratory (Derek Walsh, PhD. Thesis 1999) indicate that following BrdU treatment, the protein level and activity of eIF-4E, one of the rate limiting factors in translation of mRNAs with complex 5' untranslated regions (5'-UTR), is increased. This increase provides for a possible explanation for an increase in Ep-CAM translation following BrdU treatment.

4.2.3 ALTERATION IN FOCAL ADHESION PROTEINS FOLLOWING INDUCTION OF Ep-CAM BY BrdU.

Ep-CAM is believed to be a Ca^{2+} independent cell-cell adhesion molecule, which is involved in cell sorting. *In vitro* studies of cells transfected with Ep-CAM and non-transfected cells show that when mixed the two cell populations will aggregate separately (Litvinov *et al.*, 1994). Studies investigating the differentiation effects of the lectin *Vicia faba* in the colon cancer cell line LS174T showed that Ep-CAM was a key regulatory molecule in the promotion of differentiation (Jordinson *et al.*, 1999). These *in vitro* observations have been reflected *in vivo* studies which implicated Ep-CAM as important in the development of human fetal pancreas (Cirulli *et al.*, 1998).

This homophilic cell-cell sorting by Ep-CAM is achieved by a disruption of cadherin (e.g. E-cadherin) mediated cell-cell adhesion. Litvinov *et al.*, (1997) proposed the following model in which Ep-CAM-mediated disruption of adherens junctions was due to a redistribution of E-cadherin on the cell surface, rather than a down-regulation. This redistribution of E-cadherin, and hence disruption of its function, by Ep-CAM was via alterations in the focal adhesion proteins involved in binding E-cadherin to the cytoskeleton (figure 1.4.1). Specifically the focal adhesion proteins α-catenin, and β-catenin were down-regulated.

It was decided to investigate if the induction of Ep-CAM by BrdU induced such changes in focal adhesion proteins, α-catenin and α-actinin, in DLKP and A549.

The expression of Ep-CAM following BrdU exposure did induce a down-regulation of α-catenin in both DLKP and A549 that were treated for 21 days (figure 3.1.7). The level of decrease is quite impressive with α-catenin levels in both cell lines at half their original level following 7 days of 10μM BrdU treatment. The level of detectable protein continued to decrease over the 21 days of BrdU treatment, so that by day 21 only a very low level of α-catenin could be detected. This result is in agreement with the model proposed by Litvinov *et al.* (1997) which proposes that α-catenin levels decrease in response to Ep-CAM expression. This reduction of α-catenin levels would

disrupt E-cadherin binding to the actin cytoskeleton, and hence cause its redistribution on the cell surface.

The expression level of α-actinin was also investigated in DLKP and A549 following BrdU induced expression of Ep-CAM (figure 3.1.8). A sharp level of decrease appeared between the level of protein detected in untreated DLKP and A549 and the same cells treated for 7 days with BrdU. This decrease was repeated though perhaps not as dramatically in cells treated for 14 day with BrdU. The observed decrease in the level of α-actinin of both DLKP and A549 that occurred over the 14 days of BrdU exposure was unexpected. The study by Litvinov *et al.* (1997) upon which the model of Ep-CAM action is based did not report any decrease in α-actinin. Rather Ep-CAM is believed to interact with the actin cytoskeleton via α-actinin. It possesses two potential binding sites on its cytoplasmic tail for the binding of α-actinin (see figure 1.5.2) (Balzar *et al.*, 1998).

It would be interesting, in further work, to examine mRNA levels for α-catenin and α-actinin, and to determine if the down-regulation observed is at the transcriptional or post-transcriptional levels.

While disruption to E-cadherin binding is often seen as a prelude to metastatic events in cellular physiology, its down-regulation is also considered a normal element during development and differentiation (Christofori and Semb, 1999). One way E-cadherin function is known to be regulated is via the Rho family of small GTPases. These molecules regulate cellular processes by the phosphorylation of key regulatory proteins, members of this family include RhoA, Cdc42 and Rac1, which are known to be involved in cell shape, cell growth and cell polarity (Tapon and Hall, 1997). Modulation of E-cadherin mediated cell-cell adhesion can occur via Cdc42 and Rac1 phosporylation of an intermediate target molecule IQGAP1, which competes for α-catenin binding with β-catenin. This causes a reduction of α-catenin in the focal adhesion anchoring E-cadherin to the actin cytoskeleton, which subsequently causes E-cadherin to be localised on the cell membrane (Kuroda *et al.*, 1998). It can be speculated that Ep-CAM modulates E-cadherin utilising this family of signalling molecules.

Disruption of E-cadherin-mediated cell adhesion can also involve β-catenin. This mediation involves the phosporylation of tyrosine on β-catenin by the non-receptor tyrosine kinase Src, leading to the disassembly of the cadherin-catenin complex (Behrens *et al.*, 1993). β-Catenin is an interesting protein as it is extremely multifunctional with roles in cell adhesion (Peifer, 1995), activation of transcription factors (Molenaar *et al.*, 1996), and it is possibly involved in signal transduction via the EGF pathway (Hoschvetzky *et al.*, 1999) and WNT pathways (Molenaar *et al.*, 1996). It is hypothesised that disruption of E-cadherin may lead to an increase in free β-catenin which may translocate to the nucleus and activate members of the TCF/LEF-1 family of transcription factors. A target for up-regulation by this family is the proto-oncogene *myc* (He *et al.*, 1998). Such an increase in Myc protein levels has been observed in DLKP and A549 following BrdU treatment (Derek Walsh, PhD. Thesis 1999). Thus it is possible to contemplate that induction of Ep-CAM may have a significant influence on cell differentiation in DLKP and A549.

4.2.4 INDUCTION OF mRNA TRANSCRIPT FOR GA733-1 IN BrdU TREATED DLKP.

The GA733-1 gene is highly homologous to the gene for Ep-CAM (GA733-2). It is an intronless gene suggesting that it is due to a retrotransposition (Linnenbach *et al.*, 1989). The mRNA transcript of GA733-1 codes for a 35.7 KDa protein (Szala *et al.*, 1990). Little is known about the expression of this protein product. Limited northern blot data indicates that the GA733-1 mRNA and Ep-CAM mRNA is expressed differentially (Szala *et al.*, 1990).

RT-PCR analysis of untreated DLKP did not show any detectable transcripts for GA733-1 (figure 3.1.6). Following treatment with 10μM BrdU, however, mRNA for GA733-1 was detected in DLKP after 7 days. The level of this mRNA increased slightly after 14 days, at which point a plateau appeared to be reached with no discernible increase in cells treated for 21 days. This contrasted sharply with the results obtained for A549 where no mRNA for GA733-1 was detected in pre- and post- BrdU treated cells.

It appears that GA733-1 transcription is induced differently following 10μM BrdU treatment in DLKP and A549. It could be speculated that in DLKP, BrdU induces a promoter or removes a suppresser of transcription for GA733-1, while in A549 this change in transcription is blocked by a failure to induce such changes. This may be due to the difference in the differentiation status of these cells (i.e. A549 is more differentiated then DLKP), hence certain genes may be under different degrees of control. Furthermore, it suggests that Ep-CAM and GA733-1 protein expression is controlled via different mechanisms (at least in the cell line, DLKP), the former being post-transcriptional while the latter is transcriptional.

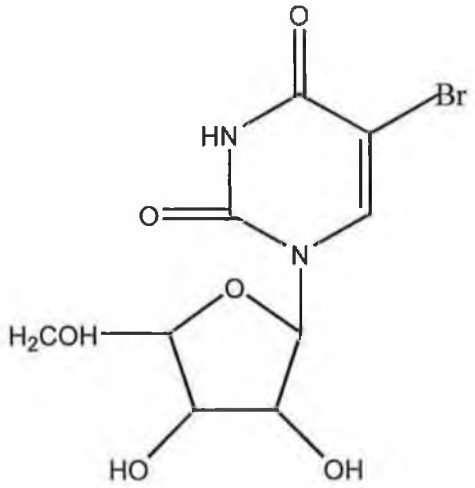
Investigation into the protein expression of GA733-1 was hindered by the lack of a commercially available antibody. After several attempts an antibody to the GA733-1 protein was sourced and this was still being waited upon at the time of writing. It was not known if the antibodies used for Ep-CAM detection were able to cross-react with

GA733-1 protein. However, protein bands with molecular weight lower than Ep-CAM were detected but these were regarded as background bands. Balzar *et al.* (1998) obtained a similar pattern of lower molecular weight products in their experiments, which they regarded as breakdown products of Ep-CAM.

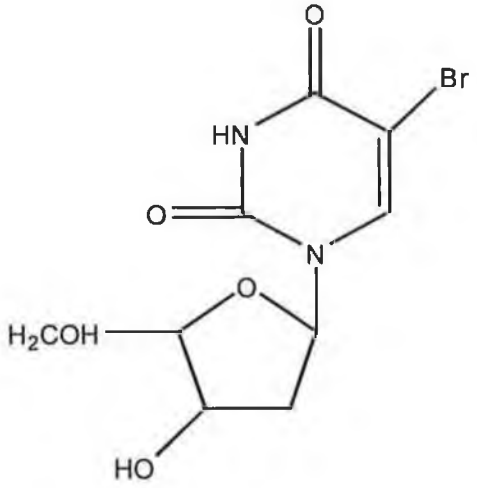
4.3 INVESTIGATION OF THE EFFECTS HALOGENATED THYMIDINE ANALOGUES HAVE ON THE DIFFERENTIATION OF DLKP AND A549.

The halogenated thymidine analogue 5-Bromo-2'-deoxyUridine (BrdU) is known to stimulate or inhibit the differentiation of different cell types. McBride *et al.* (1999) demonstrated that the differentiation-inducing effects of BrdU on DLKP and A549 were not the result of simple toxic exposure, as adriamycin, for example, did not produce the same effect.

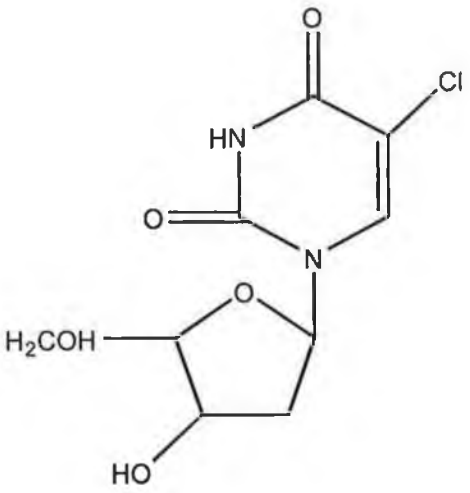
The exact mechanism by which BrdU exerts its differentiation-modulating effects is unknown though incorporation in DNA would seem to be critical (O'Neill and Stockdale, 1974). It was thus decided to investigate the ability of other halogenated thymidine analogues to induce differentiation in DLKP and A549. For this study it was decided to utilise the expression of cytokeratin (CK) -8, -18, and -19, Ep-CAM, and β_1 integrin as markers of differentiation in the two cell lines. The halogenated analogues of thymidine chosen for comparison with BrdU were 5-Fluro-5-deoxyUridine, 5-Chloro-2-deoxyUridine, and 5-BromoUridine (their structures are shown in figure 4.3.1). Preliminary experiments were performed to establish toxicity profiles of each analogue, and from these results concentrations were selected that would allow a 75% or greater survival rate.



5-BromoUridine



5-Bromo-2-deoxyUridine



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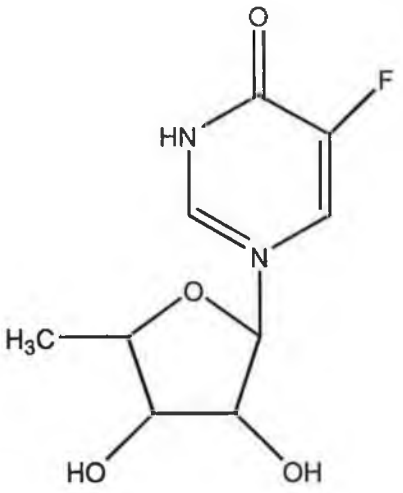

5-Fluro-2-deoxyUridine

Figure 4.3.1 Structures of halogenated thymidine analogues used for differentiation studies.

4.3.1 THE EFFECTS OF 5-FLURO-5-DEOXYURIDINE ON THE DIFFERENTIATION STATUS OF DLKP AND A549.

The toxicity profile for 5-Fluro-5-deoxyUridine (5,5'-FdU) revealed that it was particularly cytotoxic (figure 3.2.1). A concentration of 2μM was selected as the optimum concentration to allow 75% survival in both DLKP and A549.

The change of morphology in both cell lines following 7 days of treatment with 5,5-FdU was investigated (figures 3.2.3 and 3.2.4). Both cell lines exhibit a reduction in growth compared with untreated cells, and the cells adopted a more flattened morphology, though not as stretched as those cells treated with BrdU. It was noted that DLKP seem to grow in loose colonies following treatment with 5,5'-FdU, whereas A549 cells were inclined to grow as single cells.

Immunocytochemical analysis was conducted to investigate if the observed alterations in morphology were accompanied by changes in marker protein expression in 5,5'-FdU treated DLKP and A549. CK-8 expression appeared to be induced in DLKP (figure 3.2.5 C) following 7 days treatment. Similarly, in A549 treated with 2 μM 5,5'-FdU an increase in CK-8 was observed (figure 3.2.6 C). A comparable increase occurred in the expression of CK-18 in both DLKP and A549 (figure 3.2.9 C and 3.2.10 C). The expression of CK-8 and CK-18 are associated with early epithelial differentiation (Daly *et al.*, 1998), hence their induction in DLKP is indicative of an early stage in the differentiation pathway. Both cell lines also exhibited an increase in CK-19, which can partner CK-8. This increase is often used as a marker for type II pneumocytes (Paine *et al.*, 1995). The expression of these cytokeratins appear to match the expression patterns observed for BrdU-treated DLKP and A549. To quantify the changes in protein expression, western blot analysis was performed. Western blot analysis of CK-8 expression in A549 treated with 5,5'-FdU showed a strong increase in protein expression after 7 days treatment, which further increased after 14 days treatment (figure 3.2.7). The observed increase appeared to be greater then that seen after BrdU treatment. Similar increase in expression was observed for CK-18 (figure 3.2.11) and CK-19 (figure 3.2.15) expression following 5,5'-FdU

treatment. These also showed greater expression than BrdU treated cells. RT-PCR analysis lead to the conclusion that the induction of CK-8, CK-18, and CK-19 by 5,5'-FdU was at a post-transcriptional level

DLKP appears to exhibit an increase in β_1 integrin expression and an induction in Ep-CAM expression. The observations made for β_1 integrin and Ep-CAM induction 5,5'-FdU treated DLKP are similar to those made in BrdU treated DLKP.

Immunocytochemical analysis of A549 appeared to indicate that β_1 integrin expression was reduced and no induction of Ep-CAM occurred. However, western blot analysis showed an increase in β_1 integrin and an induction of Ep-CAM expression. Unfortunately, due to time constraints repeat experiments with fresh treatments were not possible to clarify this anomaly. RT-PCR analysis of A549 did not indicate any significant alteration in Ep-CAM mRNA levels indicating that protein induction was at a post-transcriptional level.

4.3.2 THE EFFECTS OF 5-CHLORO-2-DEOXYURIDINE (CdU) ON THE DIFFERENTIATION STATUS OF DLKP AND A549.

The ability of 5-Chloro-2-deoxyUridine (CdU) to induce differentiation was also investigated. Initial investigation into the toxicity of CdU (figure 3.2.2) suggested that the ideal concentration for a 75% survival rate in DLKP was 10μM and for A549 was 30μM. It was discovered subsequently that cumulative exposure to CdU was especially cytotoxic to A549. Thus a concentration of 10μM was chosen as being less cytotoxic to A549 over the treatment periods.

DLKP and A549 treated with CdU for 7 days were investigated for morphological change. Both DLKP and A549 became very flattened and stretched (figures 3.2.3 G and H, and 3.2.4 G and H). The observed alteration in morphology after CdU treatment was very reminiscent of the changes exhibited in DLKP and A549 after BrdU treatment.

Analysis of the selected marker proteins by immunocytochemistry revealed that CK-8, CK-18, and CK-19 were induced in DLKP following CdU treatment. In particular, CK-18 was strongly expressed in DLKP. Assessment of the immunocytochemistry results in A549 was difficult due to the increase in cell size. It appeared that CK-8 and CK-18 expression was increased following CdU treatment of A549. An increase of CK-19 in A549 post-CdU was also suggested.

Since immunocytochemistry is only qualitative, western blot analysis was performed to obtain a more quantitative result for changes in expression in A549. Unfortunately, due to the cumulative toxicity it was difficult to obtain a sample for 14 days treatment. The western blot analysis confirmed that 7 days of CdU treatment increased the expression of CK-8 (figure 3.2.7), CK-18 (figure 3.2.11), CK-19 (figure 3.2.15). The level of increase observed for each of the cytokeratins in A549 after 7 days CdU treatment was comparable to the level of increase in cytokeratins in A549 after 7 days BrdU treatment. Treatment with (10μM) CdU induced Ep-CAM and β_1 integrin protein expression.

RT-PCR analysis of 10μM treated A549 indicated that the mRNA level for CK-8, CK-18, CK-19, and Ep-CAM were not altered. This leads to the conclusion that CdU induces these proteins at a post-transcriptional level.

4.3.3 THE EFFECTS OF 5-BROMOURIDINE (5-BUr) ON THE DIFFERENTIATION STATUS OF DLKP AND A549.

The toxicity profiles for 5-BUr in both DLKP and A549 revealed that it did not appear to be very toxic. A concentration of 70μM was chosen for the differentiation experiments as being the most practical. Morphological studies for DLKP and A549 treated for 7 days with 5-BUr (figures 3.2.3 and 3.2.4) did not reveal any obvious alterations in morphology.

Immunocytochemical analysis of DLKP treated with 70μM 5-BU for 7 days appeared to show no induction of CK-8 (figure 3.2.5 E) and CK-19 expression (figure 3.2.13 E). There appeared to be a very slight induction in CK-18 in DLKP. Treatment of A549 for 7 days with 5-BU did not reveal any alteration in CK-8, CK-18, CK-19, β1-integrin and Ep-CAM expression. Western blot analysis for A549 did not appear to show any obvious increases in CK-8 (figure 3.2.7), CK-18 (figure 3.2.11), and CK-19 (figure 3.2.15) production, confirming the immunocytochemical observations. RT-PCR analysis did not indicate any change in mRNA levels of CK-8, CK-18, CK-19 in A549 indicating that induction is occurring at a post-transcriptional level.

4.3.4 MECHANISMS OF BIOLOGICAL AND TOXIC ACTIONS VARY ACCORDING TO HALOGENATED THYMIDINE ANALOGUE.

The halogenated thymidine analogues 5,5'-FdU and CdU induced a similar pattern of differentiation in DLKP and A549 to that observed in BrdU treatments (Tables 4.2.1 and 4.2.2), while according to markers investigated, the analogue 5-BUr did not generally induce any differentiation in either DLKP or A549, with the exception of Ep-CAM induction.

4.3.4.1 Mechanism of the biological action of BrdU.

The exact mechanism by which BrdU modulates differentiation has yet to be elucidated and a number of models exist (section 1.4.2.1). All studies published to date indicate that its stable incorporation into DNA in competition with thymidine is required. This incorporation entails BrdU being converted to bromodeoxyuridine monophosphate by thymidine kinase (O'Neill and Stockdale, 1974; Morrill *et al.*, 1980; Cortés *et al.*, 1987). BrdU incorporation into DNA occurs in a non-random fashion, with incorporation occurring into repeated nucleotide sequences (Schwartz and Snead, 1982). This consistency in the location of incorporation which may explain the reproducibility of BrdU-induced differentiation. These repeated nucleotide sequences include areas known as 'fragile sites' (Hecht *et al.*, 1988). It is envisaged that breakage's may occur at these points and these breakage's and the associated chromosomal aberrations may be associated with stepwise changes in the differentiation of a cell (Alexander *et al.*, 1992). This substitution of BrdU into DNA may also induce effects similar to DNA-intercalating agents; these alter DNA bending at either major or minor groves, and in doing so alters the structure of the promoter regions, and the affinity for DNA binding proteins. Lin and Riggs (1972) demonstrated this, when they showed that BrdU substitution in the *lac* operon allowed the *lac* supressor to bind with greater affinity. Thus, BrdU is likely to exert its effects on differentiation by alteration of an essential regulatory gene(s) that alters transcription of genes involved in differentiation (Arnold *et al.*, 1988; Rauth and Davidson, 1993). For example, in BrdU inhibition of myoblast differentiation, such an

alteration occurs with the down-regulation or complete inhibition of the key regulatory gene MyoD1 (Tapscott *et al.*, 1989; Nanthakumar and Henning 1995).

While BrdU is commonly used as an agent for the modulation of differentiation, only limited research has been performed on the ability of other halogenated thymidine analogues. Three different analogues were selected for investigation, CdU, 5,5'-FdU, and 5-BUr, each possessing a different mechanism of action.

4.3.4.2 Mechanism of the biological action of CdU.

CdU like BrdU is converted into a nucleoside monophosphate (chlorodeoxyuridine monophosphate) by thimidine kinase and then incorporates into DNA for thymidine (Cortés *et al.*, 1987). Most research on CdU has focused on its ability to generate chromosome breakage's and sister chromatid exchanges. BrdU similarly possesses this capacity to generate such chromosome breakage's, though CdU is a more potent inducer (Cortés *et al.*, 1987). For instance, CdU is reported to induce 7-8 fold more sister-chromatid exchanges than BrdU, at an equal substitution level in cultured Chinese hamster ovary cells (O'Neill *et al.*, 1983). This higher rate of sister chromatid exchanges associated with CdU may explain the cumulative cytotoxicity observed with CdU treatments of A549 and DLKP. Though only cells treated for 7 days were available for analysis, the pattern and strength of induction of proteins appeared to be similar to that observed in BrdU treatment.

In the case of the protein markers selected, the observed increase in protein level is due to changes in post-transcriptional control rather than an increase in transcription. This appears to be in contradiction with a mechanism of differentiation modulation involving alterations in DNA. Studies in our laboratory (Derek Walsh, PhD. Thesis 1999) may reconcile this apparent contradiction, as they indicate that following BrdU treatment, the protein level and activity of eIF-4E, the rate limiting factor in translation is increased. Thus, it appears that the substitution of a chloro-group in place of a bromo-group, may induce similar alterations in DNA resulting in the ultimate induction of the same marker proteins. Baker *et al.* (1979) noted that BrdU

and CdU increased interferon production in the Namalwa line of human lymphoblastoid cells.

4.3.4.3 Mechanism of the biological action of 5,5'-FdU.

The treatment of DLKP and A549 with 5,5'-FdU also induced similar changes in marker protein expression to treatment with BrdU. Although a similar pattern of expression of differentiation exists between BrdU and 5,5'-FdU treated cells, the mechanisms by which these agents are thought to exert their biological effects is quite different.

As stated previously, BrdU is incorporated into DNA and this incorporation ultimately alters the expression of differentiation related proteins. In contrast, 5,5'-FdU is cleaved by a nucleoside phosphorylase enzyme to yield 5-flurouracil (5-FU) (Armstrong and Diasio, 1980). The 5-FU generated is subsequently metabolised via several steps to yield fluorodeoxyuridine monophosphate (FdUMP) (figure 4.3.2) (Pratt *et al.*, 1994).

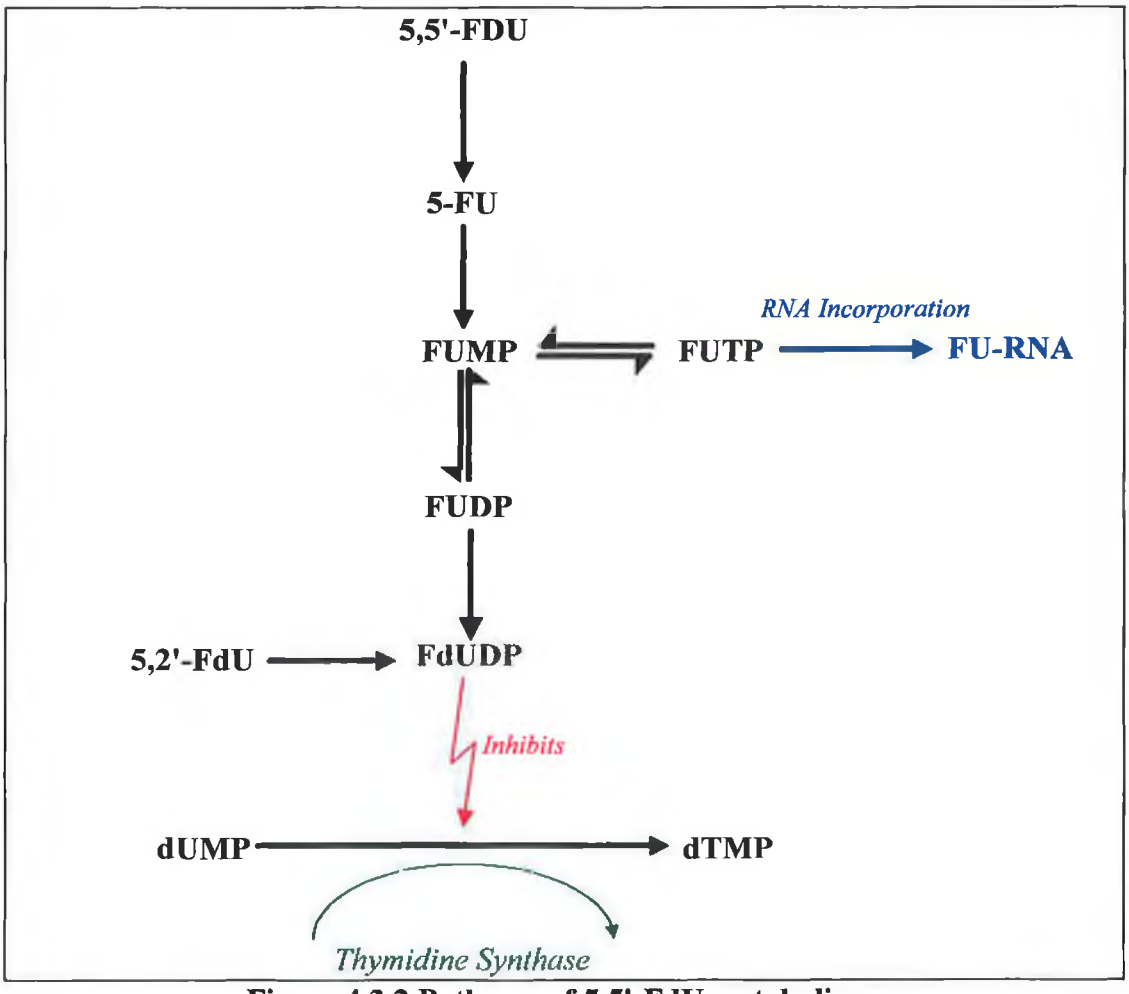

Figure 4.3.2 Pathway of 5,5'-FdU metabolism.

FdUMP binds to thymidylate synthase, forming an irreversible covalent ternary complex in which enzyme, folate cofactor, and FdUMP are bound, thus inhibiting thymidine monophosphate production and hence DNA synthesis (Pratt *et al.*, 1994). This inhibition of DNA synthesis causes cells to be delayed in the S-phase (see figure 4.2.3 for a summary of the cell cycle). The inhibition of cell growth in S-phase can induce stem cell differentiation. For example, retinoic acid induced differentiation of the embryonal carcinoma cell line PC13 to 'endoderm-like' cells is accompanied by such an increase in S-phase (Nishimure *et al.*, 1983; Mummery *et al.*, 1984). In contrast, terminal cell differentiation is associated with a prolonging of the G1-phase of the cell cycle, thus suggesting that different molecular pathways are involved in terminal and stem cell differentiation (Nishimune *et al.*, 1989). Thus, the inhibition of DNA synthesis and subsequent delay in S-phase in DLKP and A549 by 5,5'-FdU via the 5-FU metabolic pathway may trigger the molecular mechanisms required for differentiation.

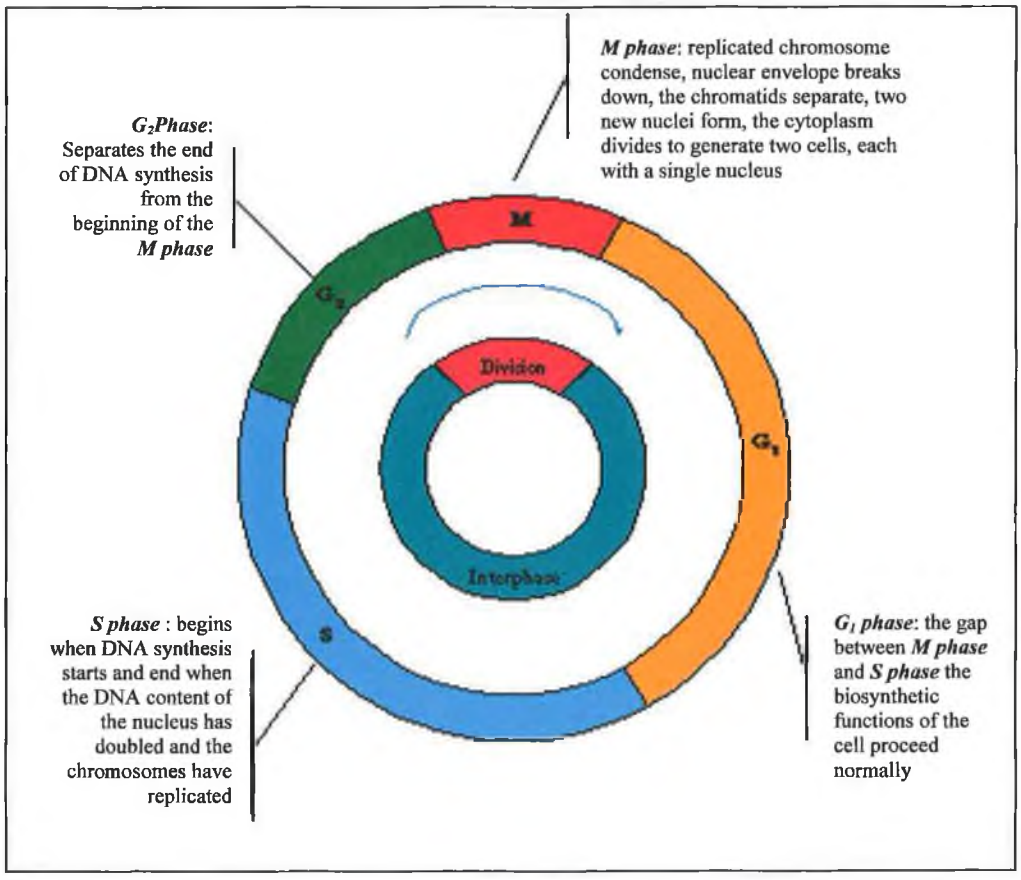

Figure 4.2.3 Summary of the cell cycle.

Though inhibition of growth may be a side effect and not a cause of differentiation. Baker *et al.* (1979) noted that BrdU increased interferon production by the Namalwa line of lymphobastoid cells while inhibiting growth. Thymidine also inhibited growth but had no effect on interferon production.

The similarity in the induction of marker proteins in A549 and DLKP by BrdU and 5,5'-FdU is thus most unusual, given their different mechanisms of action. Nishimune *et al.* (1989) reported that 5-Fluro-2-deoxyuridine (5,2'-FdU)(figure 4.2.2) induced differentiation of the teratocarcinoma cell line F9 to produce plasminogen activator, while similar treatment with BrdU failed to induce such expression. In contrast, Kidson and deHann (1990) reported that while BrdU induced differentiation in the mouse melanoma cell line BL-6 and reduced tyrosine activity, any similar reduction in tyrosine activity observed with 5-Fluro-2-deoxyuridine was due to cytotoxicity effects rather than differentiation. This suggests that in A549 and DLKP that induction of differentiation may be via a different mechanism than DNA incorporation or that the two compounds induce similar differentiation pathways via different mechanisms. Such a similar mechanism may be the inhibition of poly ADP-ribose polymerase (PARP). Pivazylan *et al.* (1992) reported that BrdU, CdU and 5,2'-FdU along with several other halogenated analogues of thymidine were good inhibitors of PARP. The PARP enzyme is located in the nucleus and catalyses the transfer of ADP-Ribose from NAD+ to target proteins (Pivazylan *et al.*, 1992). Among the list of target proteins is eEF-2, an elongation factor in the ribosomal complex involved in translation. The binding to eEF-2 of poly-ADP-ribose inhibits its activity, thereby halting protein synthesis (D'amours *et al.*, 1999)

Though the pattern of marker protein induction was similar with BrdU, 5,5'-FdU appeared to produce stronger induction. This may be due to an alternative element of 5,5'-FdU mechanism of action involving RNA. As the concentration of 5-FU increases within the cell, the 5-FU metabolic pathway may produce other metabolites most notably fluorouridine triphosphate (FUTP) (figure 4.2.2) which can become incorporated into RNA in place of UTP (Pratt *et al.*, 1994). This incorporation may alter post-transcriptional controls (e.g. specific mRNA stability) allowing the selective translation of differentiation specific mRNAs. It has been demonstrated in this

laboratory that CK-8 and CK-18 protein expression is under post-transcriptional control (McBride *et al.*, 1999) and furthermore, upon BrdU treatment this post-transcriptional control is released due to changes in eIF-4E levels and activity.

4.3.4.4 Mechanism of the biological action of 5-BUr.

5-BU is reported to affect cells most during S-phase when 5-BUr is maximally incorporated into cellular RNA (Li *et al.*, 1994). The incorporation of 5-BUr at high levels into pre-mRNA prevents splicing of the transcripts; only if the level of substitution is low (1 out of every 10 uridines) does splicing occur normally (Sierakowska *et al.*, 1989, Wansink *et al.*, 1994). From the results obtained for 5-BUr treated DLKP and A549, it would appear that the induction of differentiation by halogenated thymidine analogues requires an alteration in DNA to produce any changes in marker protein (Ep-CAM induction being an exception). However, the failure to observe any major effects following 5-BUr treatment in both cell lines may have been due to the concentration utilised not being sufficiently high to induce differentiation.

4.4 ELUCIDATION OF *IN VIVO* DIFFERENTIATION THROUGH THE DEVELOPMENT OF *IN VITRO* MODELS.

In a further attempt to develop a range of *in vitro* models to elucidate the pathways of epithelial lung differentiation *in vivo*, growth of the lung cell lines DLKP and A549 in a medium supplemented with a variety of growth factors and hormones was investigated. The establishment of primary cultures from human lung tumour samples and normal type II pneumocytes from rat lung was also undertaken. Development of such models would allow the signals, mechanisms and stages of *in vivo* epithelial lung cell differentiation to be investigated in a controlled environment. Such models may also aid in the elucidation of the putative epithelial stem cell of the lung.

4.4.1 A MEDIUM SUPPLEMENTED WITH A VARIETY OF HORMONES AND GROWTH FACTORS, INDUCES DIFFERENTIATION IN DLKP AND A549.

The lung cell line DLKP was isolated in our laboratory from a tumour histologically diagnosed as a poorly differentiated lung carcinoma. It lacks many of the ultrastructural features associated with normal differentiated cells of the lung, such as dense core granules or lamellar bodies (McBride *et al.*, 1998). DLKP also fails to express many of the normal cytochemical markers associated with differentiated epithelial lung cells for example cytokeratin proteins, desmosomal proteins, etc. (McBride *et al.*, 1998). Due to this extensive deficiency of differentiation-associated markers and the ability to induce the expression of some of these markers with the differentiation-modulating agent BrdU, it is reasonable to regard DLKP as behaving at least to some extent as a human lung stem cell (McBride *et al.*, 1999).

To date there has been no positive identification of the stem cell population in lung tissue. Prof. Emura's group in Germany has isolated a cell line (M3E3/C3) from hamster fetal lung tissue, which has properties of being a lung stem cell 'like' population. Emura and his colleagues have described the differentiation of this cell line into different functional cell types e.g. type II pneumocytes, by utilising different culture conditions and a complex hormone supplemented media (Germann *et al.*, 1993). Since DLKP shares a lot of the cytochemical features of M3E3/C3 such as lack of cytokeratin protein expression, it was decided to investigate the ability of such a hormone supplemented media (HSM) to induce differentiation in DLKP and A549. It was hoped that the complex interaction of the various constituent hormones and growth factors may replicate the *in vivo* system.

4.4.1.1 Effects of a Hormone Supplemented medium on cell morphology and the expression of differentiation markers in DLKP and A549.

It was observed that growth of DLKP cells grown in HSM for 7 days, resulted in the cells developing a squamous morphology (figure 3.3.1), similarly A549 also adopted a squamous morphology (figure 3.3.2). The number of cytoplasmic granules appeared to be increased in A549 during the 7 days of growth under HSM conditions. Since A549 is believed to be derived from a type II pneumocyte cell, the increase in the number of cytoplasmic granules may indicate an increase in the lamellar bodies associated with type II cells.

The changes in morphology were seen as indicative of possible changes in protein expression. Thus immunocytochemical analysis was performed using CK-8, CK-18, CK-19, β_1-integrin, and Ep-CAM protein expression as markers of differentiation.

Immunocytochemistry of DLKP showed expression of CK-8, CK-18 and CK-19 apparently induced following growth for 7 days in HSM. As stated previously the expression of CK-8 and CK-18 is associated with early epithelial development (Casanova *et al.*, 1995). Ep-CAM expression was also induced, suggesting that Ep-CAM may play a role in early epithelial differentiation. Western blot analysis was performed to quantify the changes in marker protein expression observed by immunocytochemical analysis. It was not possible to detect the cytokeratin expression in DLKP cells, suggesting that immunoprecipation was required.

A549 cells cultivated in HSM for 7 days appear to show slight increases in CK-8 and CK-18 expression by immunocytochemical analysis. The predominant cytokeratin in type II pneumocytes is CK-19. The detected increase in CK-19 expression was significant, since A549 is considered a type II pneumocyte 'like' cell line (Paine *et al.*, 1995; Lieber *et al.*, 1976). A549 also exhibited an increase in Ep-CAM protein expression shown by immunocytochemistry and western blotting after 7 days of growth. In A549 cells the western blot analysis confirmed a slight increase in CK-18 protein expression. A strong increase was also observed in A549 over the 14 days of growth in HSM in CK-19 expression confirming the immunocytochemical results.

The increase in cytoplasmic granules and the increase in CK-19, a marker for type II pneumocyte cells, may indicate that in this medium A549 is reverting to a more normal type II pneumocyte phenotype. Further indication came from RT-PCR analysis which showed a decrease in mRNA levels for CK-18, and a concurrent increase in the levels in CK-19 mRNA. To confirm this, further research is required into the expression functional proteins such as surfactant proteins and the ability to produce surfactant lipid. If A549 can be induced to revert to a more type II pneumocyte phenotype it may provide a useful *in vitro* model for investigation of type II pneumocyte cell biology.

Several elements of the growth medium have been identified as having possible roles in differentiation. The importance of the glucocorticoid steroids, for example, was shown important in the maturation of type II pneumocytes and Clara cells (Muglia *et al.* 1999; Beaulieu and Calvert 1981). EGF has also been identified as a possible key regulatory molecule in lung epithelial cell differentiation (Sundell *et al.* 1980; Gross *et al.* 1986). Studies investigating fetal lung development have shown localisation of EGF in the developing bronchi and around both Clara cells and Type II pneumocytes (Raaberg *et al.* 1991). Also shown to be important in lung growth and differentiation is insulin, with receptors for insulin been demonstrated on whole fetal lung and their numbers to increase during late gestation (Ulane *et al.* 1982).

The growth in HSM induced the expression of differentiation related markers in both DLKP and A549. Since it consists of a range of factors found physiologically, further analysis of the components may indicate the physiological factors controlling epithelial lung cell differentiation *in vivo*. With this hormonal treatment it would be of interest to identify what components lead to differentiation or if a combination of factors are required. For example, cell signal transduction often requires two separate events, the binding of a growth factor to its receptor and the binding of an integrin to its ECM (Cunha *et al.*, 1985). Prof. Emura and colleagues employed a range of substrata and extracellular matrix factors during their studies and these may have provided additional signals.

4.4.1.2 Assessment of the role played by individual components of HSM in the differentiation of DLKP and A549.

To attempt an identification of the elements important in HSM for the observed induction of differentiation, experiments deleting individual components from the media were performed. The effect of selective deletion was assessed after 14 days in culture by western blot.

The removal of EGF from HSM did not seem to change its ability to induce the expression of the selected marker proteins i.e. CK-8, CK-18, CK-19, and Ep-CAM during 14 days of growth. Several *in vivo* studies have suggested that EGF and the EGF family have a role in regulating early lung branching morphogenesis and with differentiation by the induction of surfactant protein C expression (Seth *et al.*, 1993, Warburton *et al.*, 1992). Another physiological role for EGF in lung tissue is the induction of proliferation in mature type II pneumocytes (Haigh *et al.*, 1989; Raaberg *et al.*, 1992). In contrast to these reports of EGF being a positive modulator of lung development, McCormick *et al.* (1995) reported that regulation of differentiation in the form of induced alkaline phosphatase expression, was inhibited by EGF. This suggests that EGF may regulate different genes, and that this regulation is both time and co-signalling molecule-dependant.

Surprisingly, the removal of hydrocortisone or oestrogen from the HSM resulted in a slight additional induction of CK-8, CK-19, and Ep-CAM protein expression in A549. This suggests that the steriod hormones are damping the inducing effect of other components in HSM. According to Spiers *et al.*, (1991) the effect of glucocorticoid steriods on induction of alkaline phosphatase in A549 is very limited, and this may be due to a lack of distal elements of the glucocorticoid receptor system (Ballard *et al.*, 1978).

In contrast, the deletion of insulin caused a profound decrease in the ability of HSM to cause induction of differentiation in A549. This correlates with reports of insulin being important in differentiation and development in other tissues, for example in the induction of breast secretory epithelium (Takahashi *et al.*, 1991; McCormick *et al.*, 1995) also reported that insulin, along with interferon-α and interleukin-6, induced

alkaline phosphatase expression in A549. Insulin is reported to be able bind to insulin-like growth factor receptors (IGF-R), though with a lower affinity than the insulin-like growth factors. It is possible that insulin in this system is exerting its influence by binding and signalling through the IGF-R pathways. *In vivo* studies have demonstrated that the IGF family, their binding proteins and their receptors, are expressed in both rodent and human fetal lung in a differential manner (Lallemand *et al.*, 1995). For example, the insulin growth factor binding protein-2 showed strong gene and protein expression in the fetal lung epithelial alveoli and airways cells (Klempt *et al.*, 1992). It has been suggested that the physiological role of IGF-signalling is to facilitate the signalling of other molecules. For example, IGF-1R signalling is required for the mitogenic and transforming activities of the EGF receptor (Coppala *et al.*, 1994). It is of interest that insulin was the only factor whose deletion resulted in a decrease in the ability of HSM to induce differentiation.

The deletion of cholera toxin from HSM resulted in a large induction of CK-19 and Ep-CAM expression, suggesting that this component had a damping effect on the induction of proteins. Cholera toxin stimulates the adenylate cyclase pathaway to induce its effects.

It appears from the deletion experiments that components such as hydrocortisone, oestrogen and cholera toxin all inhibit strong induction of the selected marker proteins. This assessment may be a bit simplistic as removal of cholera toxin allowed a very strong increase in protein expression, even though oestrogen and hydrocortisone appeared to also suppress protein induction. It may be speculated that individual components stimulate various elements required for differentiation, which then interrelate with each other. The differentiation induced by HSM is different from that induced by the halogenate thymidine analogues as it is more physiologically relevant. Furthermore RT-PCR analysis showed HSM induced changes in the levels of CK-18 and CK-19 mRNA, suggesting transcriptional control unlike the thymidine analogues which seem to act at a post-transcriptional level.

4.4.2 PRIMARY CULTURE OF LUNG CARCINOMAS.

Though establishment of tumour cell lines from solid human tissue appears relatively simple, reports in the literature are usually on singular successes. Thus, the successful routine establishment of cell lines from primary cultures of carcinomas is still a distant and elusive goal. The development of such routine cultures would potentially allow clinicians to design optimum therapeutic schedules and scientists a better understanding of the biology of tumour growth.

The principal aim of this investigation was to evaluate the different techniques for generating primary cultures from human lung carcinomas, and to develop the foundation for *in vitro* models to study differentiation in such cells.

This project analysed a total of 52 lung tumour samples, mainly from lobectomy patients. Most produced epithelial cells that grew/survived *in vitro* for short periods only before dying out or becoming overgrown with fibroblasts. Unfortunately, no cell lines were generated, suggesting that primary tumours are not necessarily a good source of material for cell line generation. If one investigates the literature it is found that most success has been with marrow aspirates and effusions from patients with metastatic lesions (Carney *et al.*, 1985). It may be that the more malignant/metastatic a cell is the more it can adapt to the artificial *in vitro* environment of cell culture. Also, solid tumours are often very necrotic and only the leading edge is viable. It is optimistically estimated that only 10-20 % of cells in a large tumour mass are viable (Leibovitz 1986). The most successful culture obtained was from a malignant melanoma of the oesophagus (WS-111)(see figure 3.4.5). The ultimate failure of this culture may have been due to an unsuitable *in vitro* culture environment.

The work to date shows that primary tumours are not necessarily an ideal tissue source for cell lines. However, it could be quite successful as a source for short-term growth of epithelial cells from tumours.

In evaluating an optimum technique that would allow cultivation of epithelial tumour cells with little interference from stromal cells, i.e. fibroblasts, it was decided to

concentrate on enzymatic versus explant techniques. It was found that the explant method was no more successful than the enzymatic methods in either cultivation of epithelial tumour cells or in the reduction of fibroblast cells, as also noted by Leibovitz (1986).

In order to investigate if the enzymes used to disaggregate the tissue sample could adversely affect the ability of epithelial cells to grow in culture, two different types of enzymatic disaggregation were evaluated. One involved a mix of collagenase, dispase, and pronase E and the other used just trypsin. Neither method seemed to confer an advantage to the isolation and generation of epithelial colonies, nor was any major difference noted in the level of stromal cells.

When generating primary by enzymatic means it has been suggested that the clumps that are retained on the 40 and 100μM mesh cell strainers may be the best source for generating epithelial cell cultures and cell lines. Mc Bain *et al.* (1984) reported a 33% success rate for generating colon carcinoma cell lines from such cell clumps. In the procedure utilised here for generating primary cultures, only a coarse filtering was employed with a stainless steel tea strainer in order to filter out very large fibrous tissue pieces. Thus the cell suspension plated contained clumps of cells. A future study looking at the culture of such clumps isolated using 40 and 100 μm mesh cell strainers may be a more successful option for the generation of cell lines, or in the general culture of epithelial cells from tumours.

When generating primary cultures from lung carcinomas an added complication is the wide variety of cell types from which the carcinoma can arise, as 40 or more different cell types occur within the lung (Crapo *et al.*, 1982). Given this complexity, the development of optimal growth conditions is difficult. The development of optimal culture conditions is important in primary culture as both growth factors and integrins can determine cell proliferation, differentiation and apoptosis. Hence, the identification of a good general culture environment with equal emphasis on media and ECM would be important in any future primary culture work.

If the generation of cell lines is deemed to be important two methods which could be evaluated for success are the transformation of cells by SV40, or the passaging of isolated cells in nude mice/rats several times prior to primary culture.

4.4.3 *IN VITRO* DIFFERENTIATION OF FIBROBLAST CELLS.

This work arose from the serendipitous discovery that fibroblast-like cells isolated during primary culture of lung carcinomas were positive for cytokeratin. The definition of a fibroblast is a cytokeratin-negative, vimentin-positive cell, and this expression pattern is considered a marker for such non-epithelial cells. Epithelial cells are identified as being cytokeratin-positive, and are negative for vimentin *in vivo*. However, once in an *in vitro* environment epithelial cells will quickly begin to express vimentin.

The results obtained from fibroblast cells obtained in the primary culture of lung carcinoma and 3 normal fibroblast cell cultures obtained from external culture collections are at variance with this criterion of being a cytokeratin negative cell.

It should be noted that the pattern of expression was not consistent across all the cells investigated. All cells were positive for pan-cytokeratin but varied in their expression for the specific cytokeratins 8 and 18, perhaps suggesting that other cytokeratins may be expressed besides 8 and 18, which are considered the early cytokeratin genes during *in vivo* development. As described in section 1.2.1. cytokeratin 19 can interact with the vimentin cytoskeleton, thus suggesting that in some cases the cytokeratin detected is not involved with a true cytokeratin cytoskeleton.

Since the cells were fibroblast-like only in morphology, it was important to investigate other antigenic markers to determine the true identity of the cells. One such antigenic marker was proly-4 hydroxylase, which is considered a fibroblast marker (Dako). Immunocytochemical staining for this antigen indicated that the cells were fibroblasts. However, as staining was detected in the epithelial cells A549 and DLKP and the haemopoietic cell line, HL-60 (data not shown) the reliability of using this antibody to define fibroblasts is questionable. Studies of normal lung tissue sections have also shown that the β-subunit of proly 4-hydroxalse can be selectively localised in type II pneumocytes (Kasper *et al.*, 1994).

However, some studies have shown that under certain conditions fibroblasts can be

induced to express cytokeratin. In a study by Giudice and Fuchs (1987) the transfection of epidermal keratin genes into fibroblasts was performed. It was found that expression of foreign type II keratin could trigger the expression of endogenous type I keratin. This induction was unidirectional i.e. type II keratin could stimulate endogenous type I, but type I keratin could not stimulate production of type II keratin. In the absence of type II keratin the type I keratin appears to associate with the vimentin network. Knapp and Franke (1987) found that in transformed lines of non-epithelial origin that rare cells emerge spontaneously that can synthesise cytokeratin -8 and -18. They found that in SV-40 transformed fibroblasts the cytokeratin-18 gene was constitutively transcribed, but broken down rapidly without its partner cytokeratin-8. These transformed fibroblasts were abundantly positive for vimentin and negative for other epithelial markers such as desmosomal protein and desmoplakin. The coexpression of specific acid and basic cytokeratins can be induced in teratocarcinoma-derived fibroblasts treated with 5-azacytidine (5-azacytidine methylates DNA bases) (Darmon, 1985). Also transient cytokeratin 19 positive fibroblast cells have been observed *in vivo* during the development of the periodontal ligament of the rat molar tooth. This expression of cytokeratin was believed to be in response to mechanical loading (Webb *et al.*, 1996).

Although the studies to date seem to indicate that the cells are fibroblast cells it would be desirable to investigate other antigens to further confirm this finding. Hence, by investigating the expression of tropomyosin, and creatine phosphokinase, as markers for smooth muscle, along with EP-16, desmoplakin, and involcurin, as epithelial markers one should be able to confirm the identity of the cells. By using immunoglold labelling in conjugation with electron microscopy it should be possible to investigate the ultrastructure of the filaments to confirm that they are true cytokeratin filaments and to further identify the exact cell type. It would also be desirable to perform western blot analysis to confirm the identity of the antigens by their molecular weight, as the antibodies may be cross-reacting with some other protein type.

Thus the question arises as to why do these cells express cytokeratin? It is possible to create a number of theories to explain this unusual phenomenon. Knapp and Franke, (1987) reported the rare spontaneous synthesis of cytokeratin in transformed cells of non-epithelial origin. This suggests that perhaps a very low percentage sub-population

of fibroblast cells might express cytokeratin and during routine cell culture it is possible that these cells are selected out. In turn, this leads to the view that fibroblasts are not the homogenous stromal cells as traditionally believed.

An alternative theory is that the *in vitro* situation mimics either a type of wound healing or stress on the fibroblast cells inducing cytokeratin expression. This theory gains some tentative evidence from two observations. During wound repair fibroblasts change to myofibroblasts with an accompanying expression of α-smooth muscle actin (Zhang *et al.*, 1996). The *in vitro* seeding density of fibroblasts can trigger this differentiation from fibroblast to myofibroblast and *vica versa*. A third theory is that the *in vitro* culture of fibroblast cells in some way causes an inappropriate expression of cytokeratin. Studies have shown that the mRNA for keratin 18 is constitutively transcribed in fibroblasts, though no protein expression occurs, as its partner cytokeratin 8 is not transcribed (Knapp and Franke 1987). Thus it is possible that *in vitro* cultivation in some way loosens the regulatory control of cytokeratin 8 and in doing so allows cytokeratin filaments to form.

To identify which, if any, of the above theories are valid, the following experiments are suggested. By performing dilution cloning, it should be possible to identify if a subpopulation exists that is keratin-positive and is being inadvertently selected for, during culture.

The relationship between fibroblasts and myofibroblasts is unclear and their relationship in repair of injury and control of the mechanism of cytoskeletal protein expression uncertain. Myofibroblasts have been found in a number of normal and pathological conditions. Myofibroblasts are essential to wound contraction and healing. Myofibroblasts differ from fibroblasts by expressing stress fibres, which contain smooth muscle α-actin. The myofibroblast cells are larger than fibroblasts and contain more cellular protein. Upon the completion of healing myofibroblasts disappear. Studies have often found cells that express the features of both fibroblasts and myofibroblasts (Masur *et al.*, 1996). These cells often appear to be larger, have a slower growth rate, and stain positive for α-smooth muscle actin. A number of studies exist in which the expression of α-smooth muscle actin by lung fibroblasts and

myofibroblasts is observed especially in pathological conditions such as bleomycin-induced pulmonary fibrosis and wound repair. Smooth muscle cells are mostly responsible for increased type I collagen expression in the lung. The emergence of myofibroblasts is associated with an increase in the expression of TGF-β, which is known to enhance smooth muscle α-actin expression and PDGF. In their study, Zhang et al., (1996) showed that TGF-β enhanced the expression of α-smooth muscle actin in bleomycin treated fibroblasts whereas the level of the non-muscular β-actin was the same in both cell types.

Since fibroblasts differentiate into myofibroblasts at low density culture (Masur *et al.*, 1996). The seeding of fibroblast cells at low density may induce a potential fibroblast-myofibroblast interconversion. This interconversion may affect the expression of cytokeratin and would be worth investigating. In a similar experiment to mimic wound healing, a confluent culture would be scraped and the leading edge of the cells investigated for cytokeratin expression. A positive result in the latter experiment would suggest that keratin expression was involved in some way in wound healing in tissue remodelling.

This work is unique, as the expression of cytokeratin in normal fibroblast cells *in vitro* has not been previously reported. It is possible that we have identified a new sub-population within fibroblasts or a mechanism involved in wound repair and mechanical stress.

4.4.4 ASSESSMENT OF DIFFERENTIATION IN ISOLATED RAT TYPE II CELLS BY TIME-LAPSE AND IMMUNOCYTOCHEMISTRY.

In-vitro type II pneumocytes are supposed to differentiate into type I "like" pneumocyte cells. Although this is widely accepted the exact sequence of differentiation events is poorly understood. Although reports exist on the proliferation of type II pneumocytes in various conditions, this proliferation is measured by indirect methods such as increased thymidine incorporation. As noted by Ulich *et al.* (1994) this increased thymidine incorporation is not always accompanied by an increase in cell numbers.

It was decided to investigate the proliferation directly by using time-lapse photography of isolated rat type II pneumocytes. These were plated at different concentrations in a well of a 12-well plate. No proliferation was observed in the cultures from the start of the experimental observation, 16 hours after isolation. Thus, if proliferation did occur with the isolated type II pneumocytes in the various culture conditions investigated, it transpired during the 16 hour period prior to the start of the time-lapse study. During the observation period the type II pneumocytes underwent a morphology change to become more flattened, with substantial increase in cell surface area. This morphology is indicative of a type I pneumocyte, although these cells still possessed lamellar bodies, thus demonstrating the potential danger of relying on the presence of this feature as a sole marker for type II cells. This sequence of events occurred in both high and low density cultures. Other observations from the time-lapse study would appear to indicate that higher density cultures entered apoptosis later than low density cultures, and that the low density cultures produced a lot more membrane ruffling.

To investigate if the observed changes in morphology of the isolated type II pneumocytes were accompanied by antigenic changes indicative of the cells becoming type I pneumocyte 'like' cells, immunocytochemistry was performed. Isolated rat type II pneumocytes were cultured for 8 days and immunocytochemistry performed at certain time points. The antigens investigated showed alkaline phosphatase activity which is considered a marker of type II function; the expression of surfactant protein D, one of the 4 surfactant proteins produced by type II cells;

expression of cytokeratin 19, which is the dominant cytokeratin in type II pneumocytes; and expression of cytokeratin 18, which is the dominant cytokeratin in type I pneumocytes. During the 8 days in culture a gradual decrease in alkaline phosphatase activity was observed. This was accompanied by a decrease in surfactant protein D levels. Both of these results indicate a loss of type II pneumocyte surfactant function over time. Concurrently changes in the cytokeratin cytoskeleton occurred with cytokeratin 19 decreasing over the period while cytokeratin 18 levels increased, indicating that the isolated cells were adopting a type I cytokeratin cytoskeleton. This is line with the observation of Paine *et al.* (1995).

The lack of proliferation observed may have been due to inappropriate culture conditions. It is reported that the growth factor KGF (FGF-10) is important in proliferation of type II pneumocytes (Ulich *et al.*, 1994). In the time-lapse studies conducted no proliferation was observed.

5.0 CONCLUSIONS AND FUTURE WORK.

5.1 CONCLUSIONS

5.1.1 BrdU INDUCES EP-CAM EXPRESSION IN THE LUNG CELL LINES DLKP AND A549.

❖ ***BrdU induces Ep-CAM expression.***

Ep-CAM is a homophilic cell-cell adhesion molecule, which was discovered only about 10 years ago. Treatment of the lung epithelial cell lines DLKP and A549 with the differentiation-inducing agent BrdU results in the induction of Ep-CAM. The induction of Ep-CAM protein expression by a simple, chemical differentiation-inducing agent in lung epithelial cells has not been reported previously, although it has been reported that dimethylsulfoxide (DMSO) treatment of the colonic cell line SW620 results in a down regulation of Ep-CAM (Omary *et al.*, 1992). Little is known about Ep-CAM expression in lung differentiation and development; evidence for a role in differentiation was shown in a study by Kasper *et al.* (1995) which found the main location for Ep-CAM immunoreactivity varied within fetal lung depending on development stage. By demonstrating its presence in lung cell lines following BrdU-induced differentiation, an important indication was gained of its potential role in *in vivo* differentiation of lung cells.

❖ ***Ep-CAM protein expression is regulated at a post-transcriptional level.***

Ep-CAM regulation was demonstrated to be post-transcriptional in both DLKP and A549. The control of Ep-CAM expression has not been fully elucidated, this result demonstrates that post-transcriptional control mechanisms may regulate the expression of Ep-CAM protein during differentiation. Our laboratory has shown that eIF-4E, the rate-limiting factor in translation, is a target for BrdU induction in DLKP and A549 (Derek Walsh PhD. Thesis 1999).

❖ ***Ep-CAM induction causes a down-regulation in focal adhesion protein levels.***

Down-regulation in levels of the focal adhesion proteins α-catenin and α-actinin were demonstrated to occur following Ep-CAM induction in DLKP and A549. The down-regulation of focal adhesion proteins signifies that various signalling events altering cell physiology occurs in response to BrdU treatment.

❖ ***The Ep-CAM homologue, GA733-1 is transcriptionally controlled in BrdU treated DLKP.***

The expression of mRNA for the Ep-CAM homologue GA733-1 was induced in 10μM BrdU treated DLKP. In contrast no mRNA for GA733-1 was detected prior to, or after 10μM BrdU treatment of A549. Western blot analysis was not possible due to difficulty in sourcing an antibody to GA733-1.

5.1.2 OTHER HALOGENATED THYMIDINE ANALOGUES HAVE DIFFERENTIATION-INDUCING EFFECTS ON DLKP AND A549.

❖ ***CdU induces differentiation in DLKP and A549.***

The biological action of CdU involves conversion to a nucleoside monophosphate (chlorodeoxyuridine monophosphate) by thymidine kinase, followed by incorporation into DNA in place of thymidine. The ability of CdU to induce differentiation in DLKP and A549 was assessed by its effects on protein expression levels for cytokeratin-8, cytokeratin-18, cytokeratin-19, Ep-CAM and β_1 integrin. Studies in our laboratory have shown that these proteins are up-regulated in both DLKP and A549 following treatment with BrdU. It was demonstrated that treatment with CdU also induced up-regulation of these proteins. According to RT-PCR analysis, CdU did not induce any changes in the mRNA level for these proteins, indicating that CdU exposure alters the post-

transcriptional/translational control of these proteins in A549. The biological action of CdU is similar to that of BrdU, so it may be speculated that the similar pattern of differentiation observed in DLKP and A549 is as a result of the insertion of a halogenated nucleotide into DNA.

❖ ***5,5'-FdU induces differentiation in DLKP and A549.***

The biological action of 5,5'-FdU in contrast requires its metabolism first of all to the base 5-flurouracil and then to Fluro-deoxyUridine Monophosphate (FdUMP). FdUMP inhibits thymidylate synthase and hence inhibits DNA synthesis by limiting TMP supply. The ability of 5,5'-FdU to induce differentiation in DLKP and A549 was assessed using changes in the expression of cytokeratin-8, cytokeratin-18, cytokeratin-19, Ep-CAM and β1 integrin. Protein analysis revealed that 2μM 5,5'-FdU treatment induced a strong up-regulation of these proteins in DLKP and A549. RT-PCR analysis suggests that 5,5'-FdU treatment alters the post-transcriptional/ translational rather than transcriptional control of the expression these proteins in A549. The pattern of differentiation obtained in DLKP and A549 with 5,5'-FdU appeared similar to the pattern of differentiation that results from BrdU treatment. This suggests that BrdU and 5,5'-FdU may activate two different differentiation pathways with overlapping properties. The induction of these proteins appeared to be stronger in cells treated with 5,5'-FdU , than BrdU. This difference in the strength of induction may be due to 5-FU being incorporated into RNA, thereby changing its structure to allow greater translation efficiency.

❖ ***5-BUr does not appear to induce differentiation in DLKP or A549.***

Investigations of the ability if 5-BUr (which incorporates into RNA) to induce differentiation revealed no such induction except for a slight induction of Ep-CAM. Hence suggesting that the modulation of differentiation by thymidine analogues requires an interaction with DNA

5.1.3 THE EFFECT OF A HORMONE SUPPLEMENTED MEDIUM ON LUNG CELL LINE DIFFERENTIATION.

❖ ***A complex hormone-supplemented medium induces differentiation in DLKP and A549.***

Differentiation was induced in DLKP and A549 using a complex hormone-supplemented medium (HSM) to represent a more *in vivo* type environment. Both cells showed induced expression of Ep-CAM, cytokeratin-8, and -19 expression. It was unclear if cytokeratin-18 was induced in A549 grown in HSM. In comparison DLKP grown in HSM appeared to show a slight induction in cytokeratin-18 expression. Experiments to identify the importance of specific components in HSM revealed that the deletion of hydrocortisone, and cholera toxin from HSM cause an **increase** in induction by the modified HSM of cytokeratin-19 and Ep-CAM in A549. In contrast the removal of insulin from HSM, **reduced** the ability of HSM to induce expression of cytokeratin-19 and Ep-CAM in A549.

❖ ***Removal of specific components from HSM alters its effects on differentiation.***

The differentiation-induction of HSM is due to a range of compounds found physiologically, acting singularly or in co-operation. It is therefore reasonable to assume that the observed differentiation is due to mechanisms different from that of BrdU and the other halogenated thymidine analogues. It is possible to speculate that that cross talk of signalling between each of the components of HSM is required to induce differentiation. It has been reported that culture of a hamster fetal lung cell line in this medium, with different culture conditions induces differentiation to different specific lung types (Emura 1996). Thus the differentiation induced by HSM of DLKP and A549 may represent an *in vitro* model of epithelial lung cell differentiation *in vivo*

5.1.4 DEVELOPMENT OF *IN VITRO* MODELS OF *IN VIVO* DIFFERENTIATION.

❖ ***Preliminary development of in vitro models reflecting in vivo differentiation.***

The methods for generating primary cultures for modelling differentiation in normal lung epithelial cells and lung tumours were established. These studies revealed that long term reproducible primary cultures were difficult to obtain. Further work is required to establish more optimum culture methods for primary cultures. Preliminary studies on the growth of isolated normal rat type II pneumocytes *in vitro* showed morphological and antigenic changes over time. These changes were consistent with the terminal differentiation of type II pneumocytes into type I pneumocytes. This spontaneous differentiation may be due to the culture environment e.g. growth factors present in the serum and/or extra-cellular matrix. During the isolation of lung tumor cells cultures of fibroblasts were often established and these expressed the unusual feature of cytokeratin protein expression, which is usually epithelial-specific. This expression may be due to the *in vitro* environment inducing changes within fibroblast cells, for example mechanical stress (Webb *et al.*, 1996).

5.2 FUTURE WORK.

5.2.1 EP-CAM INDUCTION IN BRDU TREATED DLKP AND A549.

- It has been reported that Ep-CAM directs cell-cell sorting by disrupting the function of E-cadherin, a Ca^{2+} dependent cell-cell adhesion molecule (Litvinov *et al.*, 1997). The demonstration of such disruption of E-cadherin by protein and RT-PCR analysis in the lung cell lines DLKP and A549 would be of interest, as it may form the basis of a model for *in vivo* cell-cell sorting during development.
- Experiments utilising BrdU-treated lung cells in 3-D culture to investigate the role of Ep-CAM in the formation of tissue architecture would be of great interest.
- BrdU alters the post-transcriptional/translational control of Ep-CAM expression and it has been recently demonstrated in this laboratory that following BrdU treatment, the protein level and activity of eIF-4E, one of the rate limiting factors in translation of mRNA is increased. (Derek Walsh PhD thesis, 1999). Future work requires investigation of the effect of eIF-4E transfection on Ep-CAM expression in lung epithelial cells.
- Ep-CAM is believed to disrupt E-cadherin function via alteration in the focal adhesion proteins. Thus further investigation is required into these focal adhesion proteins along with others such as β-catenin which has been implicated in a wide range of functions including control of gene transcription. Also worthy of further investigation are the signalling peptides from the Rho family, which are involved in transduction of signals from a variety of cell adhesion proteins e.g. the integrin family. Further investigation is also required on the possible targets of these changes in focal adhesion and signalling proteins, for example E-cadherin. It would be instructive to transfect Ep-CAM cDNA into DLKP and A549 to investigate what elements of the differentiation pathway it may control, for example a disruption of E-cadherin function leading to increased β1-integrin expression.

- Increased expression of Ep-CAM is found in most adenocarcinomas and *de novo* expression also frequently occurs in carcinomas of squamous epithelia (Varki *et al.*, 1984, Litvinov *et al.*, 1996). Due to these changes in expression, Ep-CAM is a target for those investigating the use of monoclonal antibody-chemotheraputic drug conjugates for anti-cancer treatment, e.g. in non small lung cell carcinoma (Elias *et al.*, 1990). The ability of BrdU to enhance the expression of Ep-CAM in carcinoma cells, may make it a useful adjuvant in such treatments. Preliminary *in vitro* and *in vivo* experiments are required to test this hypothesis.
- It is necessary to investigate if the GA733-1 mRNA induction in BrdU treated DLKP is accompanied by protein expression.

5.2.2 INVESTIGATION OF DIFFERENTIATION BY OTHER THYMIDINE ANALOGUES.

- It would also be useful to investigate if a wider range of haolgenated thymidine analogues induce differentiation in DLKP and A549 e.g. 5-FluroUridine, 5-Fluro-2-deoxyUridine and 5-Iodo-2'-deoxyUridine. These investigations may lead to the design of novel compounds that promote differentiation.
- The induction of differentiation by halogen thymidine analogues may be due to a common biological action. It has been reported that the halogenated thymidine analogues inhibit poly ADP-ribose polymerase (PARP) (Pivazylan *et al.*, 1992) One of the results of this inhibition is to relieve the inhibition of the ribosomal elongation factor eEF-2, thus allowing protein translation. Thus an investigation of eEF-2 and PARP activity may reveal if such a common mechanism exists.

5.2.3 THE EFFECT OF HSM ON LUNG CELL DIFFERENTIATION.

- Further development of this medium, with additional components and different extracellular matrices may allow the development of *in vitro* models of lung function. Research into the components responsible for the observed differentiation *in vitro*, may lead to the identification of the *in vivo* mediators of lung differentiation.
- Since the components of this medium are physiologically relevant investigation is required to determine if markers specific for differentiated lung types (e.g. alkaline phosphatase and surfactant proteins for type II pneumocytes) are expressed following growth in HSM.

5.2.4 DEVELOPMENT OF *IN VITRO* MODELS OF *IN VIVO* DIFFERENTIATION.

- Differentiation studies with primary cultures of normal and tumour cells may allow an assessment to be made as to the *in vivo* relevance of the results obtained from epithelial lung cell lines.
- Comparison of the differentiation induced in A549 and DLKP by growth in HSM with the differentiation of primary cultures would allow an assessment to be made of the *in vivo* relevance of HSM induced differentiation.
- Comparison of the mechanism of differentiation induced by halogenated thymidine analogues with that of the spontaneous differentiation in primary cultures of type II pneumocytes may allow the identification of key elements of differentiation in lung epithelial cells. Identification such elements may allow the design of novel therapeutic compounds.

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